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Utilization potentials for hardwoods as raw material for biorefinery processes

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“Forschung ist, zu sehen, was schon jeder gesehen hat und zu denken, was noch niemand gedacht hat.“

- Albert Szent-Györgyi -

Abstract

German forests are facing conflicting priorities between ecological, economic and social needs. To meet these needs under the current situation of climate change, the establishment of climate stable and resilient forest structures is crucial. Hence, the forests, former mainly dominated by softwoods, are currently adapted by integrating a higher proportion of hardwoods and a diversification of tree species. The changing raw material portfolio forces the wood processing industries to adapt their production processes or to establish new utilization paths. Parallel, forest products shall play a significant role in the bioeconomy. This emerging concept for a sustainable economic system is based on renewable resources and focuses on circular and cascading utilization of materials. However, the steadily increasing demand for fuels, fibers or products like plastics is a challenging factor. With this, the production of biobased chemicals within biorefineries for example to produce polymers becomes more and more important. In Germany, wood is the main source for lignocelluloses as potential raw material for biorefineries, due to good regional availability as well as no competition towards food production. In terms of the described changes in the raw material portfolio, especially the utilization of hardwoods becomes interesting. This is even enhanced by the ambition to reduce the direct energy production from wood, which is currently the main utilization route for hardwoods. In this case the wood and the bark becomes interesting as potential raw material for biorefineries. The underlying principle of a biorefinery is the breakdown of the grown biomass structure and the utilization of the individual chemical components. Challenges are the complexity of the lignocellulosic matrix and the variability in the chemical composition of the individual hardwood species, which potentially influence the processes and thus the yield and profitability of a biorefinery plant.

This thesis investigates the first refining step of a hardwood-biorefinery. Wood chips as raw material were pretreated by steam explosion under acidic conditions (SE). This process resulted in a solid process stream (SPS) and a liquid process stream (LPS). The cellulose rich SPS was further processed by enzymatic hydrolysis (EH) to produce monomeric glucose as the main platform product. The LPS contained water soluble substances. Four different tree species were analyzed within this thesis and two scenarios were compared. The first scenario included raw material, which was debarked (db) prior to chipping and the second scenario included bark (with bark (wb)). Beech *(Fagus sylvatica)*, ash *(Fraxinus excelsior),* oak *(Quercus robur/petraea)* and chestnut *(Castanea sativa)* were chosen based on different aspects like current abundance in Germany, economical profitability or predicted climate stability and thus future availability. Additionally, oak and chestnut are not commonly used in pulping processes, mainly due to their high content of secondary extractives, which potentially could be challenging for conversion processes in biorefineries as well. Lastly, a mixed sample (debarked) with 60 % beech, 20 % ash and 20 % oak was included in the analyses.

This thesis included three different research questions, which were examined by a broad and comprehensive experimental design. The objective of the first research question was the evaluation of the usability of the species for the processes in terms of glucan conversion rate and additional species-specific utilization potentials. Further, the distribution of the different wood components within the process streams, including the minerals was investigated. In the second research question the focus was set on the influence of bark on the usability and the EH. The investigation of different factors influencing enzymatic hydrolysis and of interconnections with species-specific effects as well as options for optimization were the objectives of the third research question.

To investigate the usability and component distribution as well as the influence of bark, first the different process streams were characterized and evaluated. Component analyses of the wood chips observed species-specific proportions and compositions of sugars, extractives and

minerals. The composition was also influenced by the presence of bark, especially regarding the content of glucan and extractives. In case of the glucan content the bark effect correlated negatively with the bark proportion, resulting in lower glucan contents for ash and oak (wb) with a bark proportion of ~15 % compared to beech and chestnut (wb) with ≤ 8 %. Strong variations between the species were observed for the hot water extract content with 15 – 23 % (db/wb) for oak and chestnut compared to beech and ash with 2 – 11 % (db/wb). Therefore, the proportions of the wood components per raw material unit was identified as one aspect to describe the suitability. The SE process mainly dissolved the hemicelluloses and lead to a relative increase of the glucan content of the SPS. With glucan contents > 60 %, chestnut db, ash db, the mixed sample and all beech samples showed the most preferable composition for following EH. However, the suitability was shown to be not solely dependent on the glucan content, but is species-specific. Beech db showed the best glucan conversion rate with 51.1 % followed by ash db with 47.5 %. In contrast, for oak db and also chestnut db less than 16 % of the glucan was converted and for the mixed sample 34.7 % due to the small oak proportion. Further, the presence of bark decreased the glucan conversion rate for all species ≥ 10 %, except for beech, where nearly no effect was detected.

For the investigation of further species-specific utilization potentials and the respective bark influence the LPS was characterized. The process stream contained a high proportion of monomeric sugars 57.2 – 72.7 % mainly originated from wood hemicelluloses. Additionally, different sugar degradation products with a proportion of 8.0 – 12.2 % of the samples were observed, with acetic acid as most abundant and quantitatively varying substance. Further, levulinic acid, furfural and hydroxymethylfurfural were detected. Between 5.7 – 10.5 % of the sample were identified as lignin degradation products and species-specific extractives. To some extent, the “fingerprint” of each species was still detectable after the SE pretreatment. This was the case for the sugar composition as well as some species-specific extractives like gallic acid and ellagic acid for oak and chestnut. Compared to the hot water extracts from wood, additional substances like the lignan syringaresinol were detected for all samples or species-specific ones like the lyoniresinol (lignan) in oak. The presence of bark mainly increased the proportions of the extractives, except for ash, where it changed the composition by the presence of different fraxinol derivatives (coumarins). Regarding the distribution of the minerals within the process streams, elements like potassium, sodium, phosphorous or manganese were completely leached into the LPS during pretreatment. Silica, magnesium and calcium were partly leached, but also still present in the SPS.

To examine the influencing factors on EH, species-specific effects and options for optimization, first the particle size and substrate accessibility for enzymes were determined. Both factors had no direct influence on the suitability for enzymatic hydrolysis. Moreover, it was shown that a low enzyme dosage based on the glucan content was well suited for the analysis of species-specific effects. The non-productive adsorption of enzymes on the substrate was identified to be an important influencing factor on EH for all wood species. This effect was examined by adding the protein BSA or the non-ionic surfactant Tween®20 to the enzymatic hydrolysis. The effect of Tween20 surpassed the effect of BSA by a factor of two. Tween20 increased the conversion of beech db about 35.0 %, which equals a conversion rate of 69.0 %. For ash db the improvements were in the same range. For oak and chestnut the effect was enhanced with an improvement of > 244 %. The glucan conversion rates of oak db, chestnut db and the mixed sample were increased up to ~55 % in the Tween20 scenario. Therefore, the prevention of non-productive adsorption had a strong effect, but could not close the gap towards beech, although, a notably stronger reduction of free protein in the reaction medium was detected for oak and chestnut compared to beech and ash. Bark reduced the glucan conversion rate in correlation to the natural bark proportion of the species. Therefore, for ash and oak a notable influence and for beech and chestnut just a low or no influence was detected. For ellagic acid as species-specific substance in oak and chestnut, no inhibiting effect was observed. In

contrast, an intensive washing of the material prior to enzymatic hydrolysis increased the conversion of oak and chestnut db about 18.5 % and 30.6 %. However, the combination of the washing effect and the Tween20 effect did not increase the conversion further. Higher and lower Tween20 concentrations lead to a decreased conversion rate, which showed that the non-productive adsorption was limited to exhaustion. These results indicated that the factor responsible for the strong reduced amount of free protein for oak and chestnut, was part of the water-insoluble proportion of the SPS and differs from the non-productive adsorption.

The species oak and chestnut contain hydrolysable tannins, which are known to interact with proteins. Therefore, a novel approach was chosen to investigate how these substances react during SE and how these affect the enzymatic hydrolysis. Wood and SPS material were extracted by acetone/water (Ace/H_2O) for oak, chestnut and as reference for beech, which contains no hydrolysable tannins. Characterization of the extracts by FTIR and (pyrolysis) GC/MS showed that potentially the degree of polymerization and the structure of aromatic components changed and the amount of tannin-related substances decreased. Additionally, oak and chestnut extracts contained a higher proportion of G-lignin derivatives, which agreed with the determined S/G lignin ratios in the SPS. The Ace/H_2O extraction of the SPS prior to enzymatic hydrolysis had no effect for beech, but for oak the glucan conversion increased from 14.7 % to 28.3 % (+92.5 %). A combination of the Ace/H_2O extraction with Tween20 enhanced the conversion rate of oak further to 90.2 %. Therefore, it was shown for the first time that the combination of an Ace/H_2O extraction and Tween20 can optimize the enzymatic digestibility of oak material and makes it competitive to beech as the reference material. An observation of the free proteins in the reaction medium of the enzymatic hydrolysis revealed, that the extraction effect acts complementary to the effect of Tween20. The extraction removed compounds, which inactivate enzymes > 75kDa and the Tween20 further prohibited the non-productive adsorption.

In a nutshell, this thesis showed the importance of species-specific characteristics for the evaluation of process suitability in hardwood biorefineries, which are independent to the glucan content. Further, the species-specific “fingerprint” stays present after the SE pretreatment and enables the utilization of these individual substances. For the suitability of the species, the non-productive binding of enzymes was identified as a strong influencing factor on EH. Additionally, for the first time the role of hydrolysable tannins in the process was investigated, complementary effects of enzyme inactivation and non-productive adsorption of enzymes were shown, and the efficiency of enzymatic conversion of oak was optimized.

Zusammenfassung

Der Deutsche Wald steht im Spannungsfeld zwischen ökologischen, ökonomischen und sozialen Bedürfnissen. Um diese unter der aktuellen Situation des Klimawandels zu berücksichtigen, ist die Etablierung von klimastabilen und resilienten Waldstrukturen unumgänglich. Durch die Erhöhung des Laubholzanteils und der Baumartenvielfalt werden die zuvor von Nadelholz geprägten Wälder angepasst. Das sich ändernde Rohstoffportfolio erfordert, dass auch die holzverarbeitende Industrie ihre Produktionsprozesse anpasst oder neue Wertschöpfungsketten etabliert. Zugleich sollen Forstprodukte eine bedeutende Rolle in der Bioökonomie spielen. Dieses aufstrebende Konzept für ein nachhaltiges Wirtschaftssystem basiert auf nachwachsenden Rohstoffen und setzt auf deren zirkuläre und kaskadische Nutzung. Der steigende Bedarf nach Kraftstoffen, Fasern und Produkten wie Kunststoffen stellt allerdings eine Herausforderung dar, wodurch die Herstellung von biobasierten Chemikalien in Bioraffinerien, zum Beispiel zur Produktion von Polymeren, immer mehr an Bedeutung gewinnt. Holz ist in Deutschland die primäre Ressource für Lignocellulosen als möglicher Rohstoff für Bioraffinerien. Holz ist regional gut verfügbar und steht nicht in Konkurrenz zur Lebensmittelproduktion. Im Hinblick auf die Änderungen im Rohstoffportfolio wird insbesondere die Nutzung von Laubholz interessant. Dieser Aspekt wird durch die angestrebte Reduzierung der aktuell hauptsächlich betriebenen direkten Energiegewinnung von Laubholz gefördert. Demnach sind sowohl das Holz als auch die Rinde von Laubholz als potenzieller Rohstoff für Bioraffinerien interessant. Das zugrundeliegende Prinzip einer Bioraffinerie ist der Aufschluss der gewachsenen Biomassestruktur und die Nutzung der im Holz vorliegenden chemischen Komponenten. Herausforderungen dabei sind die Komplexität der Lignocellulosematrix und die Variabilität in der chemischen Zusammensetzung der einzelnen Laubholzarten, die möglicherweise die Prozesse und demnach den Ertrag als auch die Rentabilität einer Bioraffinerieanlage beeinflussen.

Die vorliegende Thesis befasst sich mit der Primärraffination einer Laubholz-Bioraffinerie. Die Hackschnitzel als Ausgangsmaterial wurden durch Steam Explosion unter sauren Bedingungen (SE) vorbehandelt. Dieser Prozess führt zu einem festen und einem flüssigen Prozessstrom (solid process stream = SPS; liquid process stream = LPS). Der SPS ist reich an Cellulose und wurde weiter mittels enzymatischer Hydrolyse (EH) umgesetzt, um monomere Glucose als Hauptplattformprodukt zu gewinnen. Der LPS enthält in Wasser gelöste Verbindungen. Für die Untersuchungen im Rahmen der Thesis wurden vier unterschiedliche Baumarten ausgewählt und dabei zwei Szenarien verglichen. Das erste Szenario beinhaltet Ausgangsmaterial, welches vor dem Hacken entrindet (debarked = db) wurde, während das zweite Szenario die Rinde mitberücksichtigt (with bark = wb). Die Kriterien für die Auswahl von Buche *(Fagus sylvatica)*, Esche *(Fraxinus excelsior),* Eiche *(Quercus robur/petraea)* und Edelkastanie *(Castanea sativa)* waren ein aktuell ausreichendes Vorkommen in Deutschland, die ökonomische Rentabilität oder die Klimastabilität und daher zukünftige Verfügbarkeit. Zudem werden Eiche und Edelkastanie aufgrund ihres hohen Anteils an sekundären Extraktstoffen in der Regel bisher nicht für die Herstellung von Zellstoff eingesetzt und sind daher potenziell auch für die Umsetzung in Bioraffinerien herausfordernd. Ergänzend wurde auch eine gemischte Probe (debarked) mit 60 % Buche, 20 % Esche und 20 % Eiche in die Analysen miteinbezogen.

Diese Dissertation bearbeitet drei verschiedene Forschungsfragen mit einem breiten und umfassenden Versuchsdesign. Ziel der ersten Forschungsfrage war die Evaluierung der Nutzbarkeit der Holzarten im Aufschlussprozess hinsichtlich der Glucan-Umsetzungsrate sowie art-spezifische Nutzungspotenziale. Zudem wurde die Verteilung der unterschiedlichen Holzbestandteile in den Prozessströmen untersucht, einschließlich der mineralischen Anteile.

Für die zweiten Forschungsfrage wurde der Einfluss der Rinde auf die Nutzbarkeit und die EH analysiert. Die Untersuchung verschiedener Einflussfaktoren auf die enzymatische Hydrolyse sowie Zusammenhänge mit art-spezifischen Effekten und mögliche Optimierungsoptionen waren Zielsetzung der dritten Forschungsfrage.

In einem ersten Schritt wurden die einzelnen Prozessströme charakterisiert und evaluiert, um die generelle Nutzbarkeit der Rohstoffe, die Komponentenverteilung und den Einfluss der Rinde zu untersuchen. Die Analyse der Hackschnitzel zeigte einen Einfluss der Holzart auf die Zusammensetzung und den Anteil der Zucker, der Extraktstoffe und der mineralischen Bestandteile. Die Zusammensetzung wurde auch durch die Rinde beeinflusst, insbesondere der Glucan- und Extraktstoff-Gehalt. Der Glucan-Gehalt korrelierte negativ mit dem Rindenanteil, wodurch für Esche und Eiche (wb) mit einem Rindenanteil von ~15 % ein geringerer Glucan-Gehalt ermittelt wurde als für Buche und Edelkastanie (wb) mit einem Rindenanteil von ≤ 8 %. Insbesondere der Anteil an Heißwasserextrakten (HWE) variierte stark zwischen den Proben mit 15 - 23 % (db/wb) für Eiche und Edelkastanie und 2 - 11 % (db/wb) für Buche und Esche. Die Proportionen der Holzkomponenten pro Rohstoffeinheit sind demnach ein Kriterium, um die Eignung hinsichtlich der Ausbeute zu beurteilen. Der SE Prozess löste hauptsächlich die Hemicellulosen und führte zu einem relativen Anstieg des Glucan-Gehalts im SPS. Mit Glucan-Gehalten von > 60 % zeigten Edelkastanie db, Esche db, die gemischte Probe und Buche db/wb die günstigste Zusammensetzung für die nachfolgende EH. Allerdings stellte sich heraus, dass die Eignung nicht nur vom Glucan-Gehalt abhängt, sondern art-spezifisch ist. Buche db zeigte die beste Glucan-Umsetzungsrate mit 51.1 % gefolgt von Esche db mit 47.5 %. Im Gegensatz dazu wurde bei Eiche db und auch Edelkastanie db weniger als 16 % des Glucans umgewandelt und bei der gemischten Probe 34.7 %, was auf den geringen Eichenanteil zurückzuführen war. Die Glucan-Umsetzungsrate wurde in den Szenarien mit Rinde für alle Arten um ≥ 10 % reduziert; lediglich für Buche war der Rindeneinfluss unbedeutend.

In einem nächsten Schritt wurde der LPS charakterisiert, um weitere art-spezifischen Nutzungspotenziale und den Rindeneinfluss zu untersuchen. Der Prozessstrom bestand zu 57.2 – 72.7 % aus monomeren Zuckern, welche hauptsächlich aus Holz-Hemicellulosen stammen. Zusätzlich wurden verschiedene Zuckerabbauprodukte mit einem Anteil von 8.0 – 12.2 % in den Proben gefunden, wobei Essigsäure den größten Anteil hatte und mengenmäßig am stärksten variierte. Zudem wurden Lävulinsäure, Furfural und Hydroxymethylfurfural detektiert. Weitere 5.7 – 10.5 % entfielen auf Ligninabbauprodukte und art-spezifische Extraktstoffe. Der „Fingerabdruck“ der jeweiligen Holzart war teilweise auch nach dem SE Prozess noch nachweisbar, zum Beispiel bei der Zuckerzusammensetzung und einigen art-spezifischen Extraktstoffen wie Gallussäure und Ellagsäure für Eiche und Edelkastanie. Im Vergleich zu den HWE vom Holz, wurden aber auch zusätzliche Stoffe wie das Lignan Syringaresinol in allen Proben oder art-spezifische wie Lyoniresinol (Lignan) in Eiche gefunden. Die Rinde erhöhte hauptsächlich den Extraktstoffanteil, außer für Esche, wo sich auch die Zusammensetzung durch das Auftreten von Fraxinol-Derivaten (Cumarine) änderte. Bezüglich der Verteilung der mineralischen Bestandteile in den Prozessströmen wurden Elemente wie Kalium, Natrium, Phosphor oder Mangan während der Vorbehandlung vollständig in den LPS ausgewaschen. Silicium, Magnesium und Calcium wurden teilweise ausgewaschen, waren aber auch noch im SPS vorhanden.

Für die Untersuchung der Einflussfaktoren und der art-spezifischen Effekte auf die enzymatische Hydrolyse sowie der Optimierungspotenziale wurden zunächst die Partikelgröße und die Substratzugänglichkeit für Enzyme untersucht. Für beide Einflussfaktoren konnte kein direkter Einfluss auf die Eignung für die EH festgestellt werden. Zudem wurde gezeigt, dass eine niedrige Enzymkonzentration bezogen auf den Glucan-Gehalt der Probe gut geeignet ist, um art-spezifische Effekte zu untersuchen. Für alle

Holzarten wurde die nicht-produktive Adsorption von Enzymen als wichtiger Einflussfaktor auf die EH identifiziert. Dieser Einflussfaktor wurde durch die Zugabe des Proteins BSA oder des nicht-ionischen Tensids Tween®20 zur EH untersucht. Beide Additive hatten einen positiven Effekt, wobei Tween20 die Umsetzungsrate doppelt so stark verbesserte als BSA. Tween20 erhöhte die Umsetzung von Buche db um rund 35 %, was einer Umsetzungsrate von 69.0 % entspricht. Die Umsetzung von Esche db wurde im selben Maße verbessert. Bei Eiche und Edelkastanie war die Wirkung noch deutlich stärker ausgeprägt mit einer Steigerung von > 244 %. Die Glucan-Umsetzungsraten von Eiche db, Edelkastanie db und der gemischten Probe wurden mit Tween20 auf ~55 % erhöht. Die Unterbindung der nicht-produktiven Adsorption hatte demnach einen starken Einfluss, konnte aber den Unterschied zur Buche nicht ausgleichen, obwohl für Eiche und Edelkastanie im Vergleich zur Buche und Esche eine deutlich stärkere Reduzierung der freien Proteine im Reaktionsmedium festgestellt wurde. Die Einbeziehung der Rinde reduzierte die Glucan-Umsetzungsrate in Abhängigkeit von dem natürlichen Rindenanteil der Baumarten. Demnach wurde für Esche und Eiche ein deutlicher und für Buche und Edelkastanie nur ein geringer bis kein Einfluss festgestellt. Für Ellagsäure, als art-spezifische Substanz in Eiche und Edelkastanie, konnte kein inhibierender Effekt nachgewiesen werden. Im Gegensatz dazu konnte ein intensives Waschen des Materials vor der EH die Umsetzung von Eiche und Edelkastanie db um 18.5 % und 30.6 % steigern. Allerdings konnte die Kombination aus Wasch-Effekt und Tween20-Effekt die Umsetzung nicht weiter erhöhen. Höhere und niedrigere Tween20 Konzentrationen führten zudem zu einer verringerten Umsetzungsrate, was zeigte, dass die nicht-produktive Adsorption bereits maximal unterbunden war. Demnach war der Faktor, welcher für die starke Reduzierung des freien Proteins bei Eiche und Edelkastanie verantwortlich ist, Teil des wasserunlöslichen Anteils des SPS und unterschiedlich zur nicht-produktiven Adsorption.

Die Holzarten Eiche und Edelkastanie enthalten hydrolysierbare Tannine, von denen bekannt ist, dass sie mit Proteinen interagieren. Daher wurde ein neuartiger Ansatz verfolgt, um zu untersuchen wie diese Stoffe während des SE Prozesses reagieren und wie diese die enzymatische Hydrolyse beeinflussen. Holz und das SPS Material von Eiche, Edelkastanie und von Buche, welche als Referenz keine hydrolysierbaren Tannine enthält, wurden mit Aceton/Wasser (Ace/H_2O) extrahiert. Eine Charakterisierung der Extrakte mittels FTIR und (Pyrolyse-) GC/MS zeigte, dass sich möglicherweise der Polymerisationsgrad und die Struktur der aromatischen Komponenten verändert und der Anteil an tannin-verwandten Substanzen abnahm. Zudem enthielten die Extrakte der Eiche und Edelkastanie einen größeren Anteil an G-Lignin Strukturen, was mit den ermittelten S/G Lignin Verhältnissen im SPS übereinstimmt. Die Ace/H_2O Extraktion des SPS vor der EH hatte keinen Effekt für Buche, aber für Eiche stieg die Umsetzungsrate von 14.7 % auf 28.3 % (+92.5 %). Die weitere Kombination der Ace/H_2O Extraktion mit Tween20 erhöhte die Umsetzungsrate für Eiche weiter auf 90.2 %. Damit wurde erstmals gezeigt, dass durch die Kombination der Ace/H_2O Extraktion mit Tween20 die enzymatische Umsetzung von Eichenmaterial optimiert werden kann und somit konkurrenzfähig zur Buche als Referenzmaterial wird. Die Untersuchung der freien Proteine im Reaktionsmedium zeigte zudem, dass der Extraktions-Effekt komplementär zum Tween20-Effekt agiert. Die Extraktion entfernte Komponenten, welche Enzyme > 75 kDa inaktivieren und das Tween20 unterbindet zudem die nicht-produktive Adsorption.

Zusammengefasst zeigt diese Thesis die Bedeutung der art-spezifischen Eigenschaften für die Bewertung der Prozess-Eignung für Laubholz-Bioraffinerien auf, welche unabhängig vom Glucan-Gehalt sind. Zudem bleibt der art-spezifische „Fingerprint“ auch nach dem SE Prozess erhalten und ermöglicht damit die gezielte Nutzung der holzeigenen Inhaltsstoffe. Für die Eignung der Holzarten wurde die nicht-produktive Adsorption von Enzymen als ein starker Einflussfaktor auf die EH identifiziert. Zudem wurde erstmals die Rolle der hydrolysierbaren Tannine im Prozess untersucht und komplementäre Effekte bezüglich Enzyminaktivierung und

nicht-produktive Adsorption von Enzymen festgestellt, sowie die Effizienz der Umsetzung von Eiche optimiert.

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Abbreviations

Superset	Description	Abbreviation
Samples	Acid insoluble residue	AISR
	Acid soluble residue	ASR
	debarked	db
	Liquid process stream	LPS
	Solid process stream	SPS
	Solid process stream pre washing	SPSpw
	Sweet chestnut	chestnut or Chest
	with bark	wb
Processes	Enzymatic hydrolysis	EH
	Steam explosion	SE
Chemicals	Acetone	Ace
	Acetonitrile	ACN
	Arabinose	Ara
	Bovide serum albumin (protein)	BSA
	Dichloromethane	DCM
	Dimethylformamid	DMF
	Ethanol	EtOH
	Galactose	Gal
	Glucose	Glc
	Hot water	HW
	Hydroxymethylfurfural	HMF
	Mannose	Man
	Methanol	MeOH
	N,O-Bis(trimethylsilyl)trifuoracetamide	BSTFA
	Potassium bromide	KBr
	Rhamnose	Rha
	Sodium dodecyl sulfate	SDS
	Tetramethylethylendiamine	TEMED
	Trichloro(methyl)silane	TMCS
	Trifluoroacetic acid	TFA
	Tween20	Tw20
	Water	H_2O
	Xylose	Xyl

continued ↓

Superset	Description	Abbreviation
Methods / Instruments	Brunauer-Emmett-Teller	BET
	Critical point drying	CPD
	Energy dispersive X-ray analysis	EDXA
	Fourier transform infrared (spectroscopy)	FTIR
	Gas chromatography with mass spectrometer	GC/MS
	High performance liquid chromatography	HPLC
	Inductively coupled plasma (mass spectrometry)	ICP
	Nuclear magnetic resonance (spectrometry)	NMR
	Particle size distribution	PSD
	Principle component analysis	PCA
	Refractive index detector	RI
	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis	SDS
	Solid phase extraction	SPE
	Solute exclusion	SX
	Solvent exchange drying	SED
	Ultraviolet-visible light detector	UV/Vis
	Water retention value	WRV
Enzymes	Carbohydrate binding domain (of enzymes)	CBM
	Cellobiohydrolase	CBH
	Core domain	CD
	Endoglucanase	EG
	Filter paper unit (cellulase activity)	FPU
	ß – Glucosidase	BGL
Others	Microcrystalline cellulose	MCC
	Pondus Hydrogenii	pH
	Relative centrifugal force	rcf
	Revolutions per minute	rpm
	Unit for relative pressure (pressure over atmospheric pressure)	barg

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1. Introduction

Wood is one of the most important raw materials utilized by humans since ancient times. At the Bronze Age human activities started to have a significant impact on forested areas. Wood was a resource of high demand for instance for mining, for buildings or as fuel for metal melting and salt extraction. The impact intensity thus correlated with times of cultural primes alternating with times of war or natural disasters like failed harvests or the plaque. The next wave of deforestation in Central Europe was during the middle age to promote agricultural and settlement area. With the strong population increase starting in the 16th century the demand on wood as raw material raised as well, which resulted in an extreme wood shortage due to devastation and exploitation of forested areas. In 1713, the Saxon mining engineer Hans Carl von Carlowitz first published the concept of sustainable management within the book "Silvicultura oeconomica". The basic principle of sustainable management is to balance regrowth and harvest to ensure continuous resource supply. This concept initialized an active silvicultural management with the aim to produce wood as multipurpose raw material. This included active decisions and planning on tree species, cultivation and harvesting (Sauter & Scheiding, 2023). In Germany, devastated regions were reforested by planting mainly spruce and pine, which could be cultivated on the greenfield and produce good quality wood at the same time. This resulted in mostly homogenous softwood dominated forests and the according development of a forest and wood industry focused on the utilization of softwoods, despite the fact, that Germany would be covered by beech dominated forests, according to the potential natural vegetation (Bartsch et al., 2020; Fischer, 2002). The high dependency on wood as energy source decreased during the industrial revolution in the 19th century with the large scale utilization of fossil resources like coal and mineral oil (Miletzky et al., 2020; Zamani, 2015).

Climate political events like the Kyoto Protocol in 1997 and the Paris Convention in 2015 strongly increased the awareness for climate related topics within public and set an international frame for actions against climate change. The presentation of climate change impacts based on scientific results strengthened the awareness about the importance of forest ecosystems, their preservation and sustainable utilization. One subsequent measurement was to develop adaptation strategies for German forests to meet the challenges of climate change. The development of climate resilient forests includes an increase of the hardwood proportion within the forests and a general higher diversity of tree species to distribute the risk of calamities and to benefit from species synergisms (BMEL, 2021). As a result, from 1960 to 2012 the proportion of hardwoods in German forests raised from 28 % up to 43 % (Bartsch et al., 2020; BWI, 2012). These substantial changes in the raw material portfolio lead to a focus on the developments and trends in Germany.

Parallel to this development the transformation of the current economic system towards a bioeconomy becomes more and more important. The bioeconomy follows the principles of sustainability and prioritizes the utilization of renewable resources and energy systems. The aim is to drastically reduce the dependency on fossil resources by increasing the utilization of renewable biomass and promoting a material, circular and cascading utilization over direct energy production (BMBF & BMEL, 2020). This transformation of the economic system and the changing raw material portfolio in German forests urges the wood and other industries to develop new utilization routes. This is especially important for hardwoods as half of the available hardwood volume is currently used for direct energy production, which is contrary to the principles of a sustainable bioeconomy. Additionally, the processing industries in the past were mainly adapted to softwoods due to the high availability and good processability as well as a high demand (BMEL, 2021). However, from market perspective the sustainable available amount of wood is completely subdivided between the industries (FNR, 2018). The implementation of new utilization routes thus needs a redistribution of the resources.

Common material utilization options for hardwoods are furniture and veneer production or the application in constructions. Further, birch, beech and eucalyptus are also used for the pulp and paper production (Ek et al., 2007). Another option to establish new material utilization routes would be the use of hardwood as raw material for lignocelluloses biorefineries, which target to utilize individual wood components like cellulose and lignin. These wood components can be used as platforms to synthesize for instance modified cellulose fibers or chemicals, fuels, and further value added products for the chemical, food, cosmetic or pharmaceutical industry and thus substitute fossil based products or enable the development of new, innovative products (BMEL, 2021; FNR, 2018). The term biorefinery is quite new whereas the basic principle behind it is rather established. In terms of the definition one of the oldest “biorefinery” industries might be the pulp and paper industry. However, due to the more recent use of the term and the current focus on biofuels and chemicals, research regarding pulp and paper is mostly not directly related to the term biorefinery (Wenger & Stern, 2019). However, the pulp and paper industry is a well-known example for the use of implemented disintegration processes of wood by using sulfate and sulfite pulping to produce cellulose (Berg & Guzmán, 2023). For the use of wood as raw material in biorefineries, the disintegration of lignocellulosic biomass is crucial to get access to the main cell wall constituents (Berg & Guzmán, 2023; Karimi, 2015; Miletzky et al., 2020). Over the last decades until today, there was numerous research on different disintegration processes and types of biomass. However, the implementation rate of further biorefinery processes based on lignocelluloses, other than sulfite and sulfate pulping, is very low, especially in demonstration or industrial scale. These activities are notable higher for other raw materials like starch or sugar crops (Wenger & Stern, 2019). This is mainly reasoned in the natural complexity of lignocelluloses and the high variability of the chemical composition and related properties between the individual species (Berg & Guzmán, 2023; Fengel & Wegener, 2003; Mai & Zhang, 2023; Wenger & Stern, 2019).

2. State of the art and knowledge

This chapter presents the background and current state of research and developments to point out uncertainties and gaps in research, which were targeted by this thesis. The different paragraphs are numbered continuously, but are thematically subdivided into three sections:

A. The role of silviculture and wood within the German bioeconomy and developments in wood utilization

B. Wood and bark – anatomical and chemical properties

C. The disintegration processes of steam explosion (SE) and enzymatic hydrolysis (EH) and influencing factors

A. The role of silviculture and wood within the German bioeconomy and developments in wood utilization

2.1 Development and adaptation of German forests due to climate change

German forests in combination with wood utilization already improve the German greenhouse gas balance about 11 – 14 % and are thus very important instruments to manage the impacts of climate change. A strategy for the management of German forestry was published in 1990, which targets to re-structure the forests to establish climate stable systems. These actively function as a carbon sink, maintain biodiversity and produce high valuable raw material, which stores carbon longtime. Further, climate stable forests provide frequent ecosystem services like social recovery or provision of drinking water (BMEL, 2021).

Because of historic developments and the high market demand homogenous, softwood dominated forest stands are a well-known picture in the German landscape. However these spruce and pine dominated structures are at high risk due to different threads like insect calamities (bark beetle) and extreme weather events like storms or droughts, which are directly or indirectly linked to climate change (BMEL, 2021). Currently, the proportion of damaged trees is thus very high with 75 % of the total harvests in 2020, which is mainly caused by storms and insect calamities (bark beetle) (Schmitz et al., 2022).

A higher diversity of species including genetics and a nature-near forest management are important to decrease the risks and increase the resistance and resilience of forests (BMEL, 2021). Therefore, the strategy to form climate stable forest stands targets an increase in the proportion of native hardwoods. However, this potentially leads to a lower productivity as well, because hardwoods differ from softwoods for example in the point where their crown commences, resulting in a lower proportion of stem wood (Sauter & Scheiding, 2023). Additionally, a study about the forest status showed a lower vitality of trees (soft- and hardwoods) older than 60 years, which increases the risk of damages through extreme weather events or insect calamities (BMEL, 2023). This shows that the ability to adapt to and resist effects of climate change decreases with rising age of the trees (BMEL, 2021). This points out the importance of a balanced forest management to maintain vital tree stands.

From 2012 to 2017, the proportion of softwood in forest stands decreased (56 % ➔ 55 %) and the proportion of hardwood increased slightly (44 % ➔ 45 %). Beech *(Fagus sylvatica)* is the main hardwood tree species in Germany with a total stock of 635,257 thousand m³, which increased about 10 % from 2002 to 2012. (BWI, 2012). Oak *(Quercus robur/petraea)* is the second most important hardwood species in Germany with a wood storage of 361,231

thousand m³ (BWI, 2012). The individual species of English oak (Q. robur) and Sessile oak (Q. petraea) are very similar in their properties and are not separately distinguished in timber harvesting (Grosser, 1977).

There are some modelled scenarios available, which picture the future potential vegetation. These models are often calculated for specific regions, as the forestry management is in the responsibility of the individual federal countries of Germany. In the following, the scenarios available for Bavaria are chosen as an example. The Bavarian model scenarios consider a precipitation reduction of 10 % and rising temperature from 1 - 6 C°. The model clearly shows a strong increase of oak dominated forest communities for a temperature rise up to 3 °C. However, with higher temperatures, the growing conditions change in a range, which are not existent in Bavaria to date. Within the oak species available in Germany the already common species *Q. petraea*, as well as some rare species like *Q. pubescens* or *Q. cerris* are potential future species with a predicted good climate stability. One the one side, using oak for an active forest management has potential to establish climate stable forests, to secure a continuous production of high valuable wood and is very rich in ecological benefits. On the other side, it has some challenges as well. Oak species need light forest stands to grow, unlike beech or lime, which can also grow in the shadow of other trees. This leads to a high effort to found and maintain oak dominated forests at least under the current conditions. Additionally, there are typical insect calamities known for oak stands (Fischer et al., 2018; Klemmt et al., 2018). Up to date, oak dominated forest stands account to ~ 1 Mio ha, which is 10.5 % of the forested land in Germany (BMEL, 2021). In the guidelines of the Bavarian State Institute of Forestry, the oak species *Q. robur* and *petraea* are described as species with very low to low cultivation risk for Bavaria in 2100, which is mainly based on the medium to high tolerance against drought (LWF, 2019).

Another species with some potential for the changing climate conditions is sweet chestnut *(Castanea sativa)*, which is already present in Germany, especially in the warmer regions, which are also used for wine cultivation. To date the species is not abundant in other German regions and is not included in common silviculture management plans. The species can tolerate warmer temperatures than beech and oak, but is sensitive to droughts and alkaline soils (Fonti et al., 2002). The resistance and resilience of the species seem to be well comparable with other native species. With a high growing rate until the age of 40, the wood production is notably stronger than for beech. With the correct management, the harvest of stems with a diameter of 50 - 60 cm after 60 years is possible. A strict management is also important to reduce the risk of value reduction through heart shakes (longitudinal cracks following the year rings) (Fonti et al., 2002). Chestnut wood shows valuable material properties with a high tensile strength (135 N/mm²), a moderate elasticity (E-modulus 9000 N/mm²) at the same time (Richter & Ehmcke, 2018). To reduce the rotation time further the wood can also be harvested after 25 - 30 years for utilization as palisade timber with a diameter of 20 cm, for floors, terraces or fences. Despite the benefits, there are two pathogens threatening the species (Hein et al., 2016; Lüpke et al., 2018). The first one is called ink disease caused by *phytophthora*. One way to prevent an infection is to avoid cultivation at stands with backwater or influence through groundwater. The pathogen *C. parasitica* can be quite aggressive, but there is ongoing research to tackle the fungi by hypovirulence (Peters et al., 2016).

The tree species beech *(Fagus sylvativa)* is described as a species with high tolerance against shadow and acidic soil conditions, but shows a low resistance against drought. Based on the Bavarian forest information system, the guideline describes the risk of cultivating beech still at low level in 2100. For comparison, the cultivation risk for spruce is classified as very high for most parts of Bavaria in 2100 (LWF, 2019). However, there are current studies showing that beech already shows high damages due to low water availability in some places in Germany (BMEL, 2023).

The cultivation risk of other native hardwood species like maple *(Acer platanoides or pseudoplatanus)* varies strongly within Bavaria. It is less dependent on the drought tolerance, but the species need high alkaline soils, which are rare in the east and south of Bavaria. Alkaline soil conditions are a limiting factor for the cultivation of ash *(Fraxinus excelsior)* as well, but on suitable stands, the cultivation of this species would be still possible. Ash is a remarkable tree species as it has good technological properties and can grow in meadowlands, what make the species favored for wet stands. Unfortunately, in recent years, this tree species is heavily threatened by the fungus *Hymenoscyphus pseudoalbidus* and is thus currently not suitable for cultivation (LWF, 2019). Therefore, it is hardly predictable which role this species will play in future forest stands.

Regarding the cultivation risk scenarios it has to be considered that these are based on various model calculations using different approaches or bases for the evaluation of species distribution, soil conditions and climate development and underlay some uncertainties. The climate component within the guidelines of LWF are based on the optimistic scenario B1 including a temperature rise of 2 °C until 2100. This scenario is part of the SRES scenarios (Special Report on Emission Scenarios) from 2000 of the intergovernmental panel on climate change (IPCC). However, there are new scenario families for example the RCP scenarios (Representative Concentration Pathways), which consider the effect of greenhouse gas concentrations and radiative forcing instead of socio-economic aspects (Falk et al., 2013; Kasang, 2008, 2013; Spekat et al., 2007). For Baden-Württemberg there are maps available, showing the cultivation suitability of some species applying the RCP 4.5 or RCP 8.5 scenario, which include a rise in temperature of 2.6 °C and 4.8 °C compared to the pre-industrial values. The RCP 4.5 scenario is most similar to the B1 scenario (SRES) for the radiative forcing component. Following the RCP 8.5 scenario in 2100 the model shows that there are just few places in Baden-Württemberg, which will be suitable to cultivate spruce, beech, fir or oak as leading species (FVA, 2019). A diversification of the species-portfolio and the development of a healthy and stable ecosystem is thus crucial to spread the risk of a complete malfunction of forest and thus to secure the wood supply.

2.2 Wood and biorefineries in the framework of bioeconomy

The German Federal Government defines bioeconomy as followed:

“ (…) ‘bioeconomy’ refers to the production, exploitation and use of biological resources, processes and systems to provide products, processes and services in all economic sectors within the framework of a sustainable economic system.” (BMBF & BMEL, 2020)

The German bioeconomy strategy is interconnected to 11 of the sustainable development goals of the United Nations (Agenda 2030 for Sustainable Development) and follows two main guidelines. The first one is about “harnessing biological knowledge and responsible innovation for sustainable, climate-neutral development” and the second guideline focuses on “using biogenic raw materials for a sustainable, circular economy”. Possible sources for raw materials are agriculture, forestry or marine systems as well as biogenic waste streams (BMBF & BMEL, 2020). For the bioeconomy, social, ecological and economical aspects are always interdependent (Schmitz et al., 2023).

One instrument of sustainable development is the principle of circularity. To implement a circular and sustainable bioeconomy it is necessary to cross-link all dimensions of the system, re-think the resource consumption as well as the product design, the production process and the supply chains. The closing of loops for instance by recycling is thus just a small part of the principle, which is more about retaining values by decoupling economic growth form resource consumption. One important advantage of wood, next to its renewable character, is the possibility to use the material in a cascading manner (BMEL, 2014). Wood can be used in

every structural level, retaining its value and carbon storage properties, considering the effort to transform the products into each other. Wood can be used as tree stem or sawn timber, in form of smaller particles in OSB or particle boards or even on a molecular level to use the cellulose, lignin or polyoses.

Based on the German bioeconomy strategy the national bioeconomy council developed four fields of actions with 10 defined topics and regarding recommendations for action. One defined topic focuses on wood and lignocelluloses as important renewable and local available raw material in Germany. The enhancement of existing and the development of new utilization routes, especially in the field of bioconversion and biorefinery processes are crucial to make a valuable contribution for the bioeconomy transformation. However, the future availability of these raw materials, considering aspects of climate change, soil ecology, biodiversity and carbon sink, is limited and points out the need of a smart and holistic/circular utilization of the materials. The recommendations of action, thus focus on the need to prioritizing material over energy use and the importance to establish guidelines for the utilization of wood/lignocelluloses to keep the use within the capacities of the ecosystem, next to others (BÖR, 2023).

The system of a circular and sustainable bioeconomy perfectly fits the field and recommendations of actions about wood and lignocelluloses of the national bioeconomy council. To meet the need of circularity former business strategies, which used high resource volumes to produce one or just a small amount of different products, but in high quantity are not expedient. There is a demand of business strategies, which aim to produce a variety of different products without increasing the resource consumption. This would enable a reduction of external dependence and would increase the resilience of the economic system for instance due to a higher flexibility to react on changes in market demand and thus foster resilient supply chains (Schmitz et al., 2023).

One example for a business strategy offering a wide product portfolio are biorefineries, which follow an integrated approach for example to utilize the different wood components individually to produce various products for the chemical, food, construction or pharmaceutical industry (BMEL, 2014). The concepts of biorefineries are another defined topic in the recommendations for action of the German bioeconomy council. Currently, there are few examples for biorefineries in Germany, other than pulp and paper production, but they have great potential to support the transformation of the chemical industry. The enhancement of research about possible raw materials to increase the raw material portfolio for biorefineries as well as the research and development of suitable conversion processes are some of the recommended advices of action (BÖR, 2023). These new technologies need to be able to process the high diversity of lignocellulosic biomass, but producing a uniform product portfolio at the same time (BMEL, 2014).

2.3 The concepts behind biorefineries

The U.S. department of agriculture (USDA) defines a biorefinery as a *"facility, including equipment and processes, that converts renewable biomass or an intermediate ingredient or feedstock of renewable biomass into one or more or a combination of biofuels, renewable chemicals or biobased products"* (USDA, 2019). The explanation of the term biorefinery within the paper about the recommendations of action of the German bioeconomy council is quite similar to the cited definition, but additionally includes to use the raw material source in the most holistic way possible (BÖR, 2023).

A biorefinery is classified by the raw material, the intermediates, also referred to as platforms and the respective process(es) to generate these platform(s) from the raw material within the primary refining step. The different intermediates are then further processed within the secondary refining step to semifinal or final products. In the German biorefinery roadmap, the

raw material for biorefineries are classified into four groups. The first one includes “renewable resources” from forestry or agriculture and aquatic resources, but without food or feed purpose. The second one includes “biogenic residual material from agriculture and forestry”, like leaves, black liquor or sugar beet pulp. The third one comprises “industrial biogenic residual materials”, which include waste streams from industrial processing, for instance the slurry after fermentation. The fourth group are the “biogenic waste materials”, which comprise the waste streams after the life time of a product, like used wood or used cooking oils (FNR, 2012).

The process(es) of the **primary refining** include the preparation of the raw material and the subsequent disintegration process to separate the biomass components, which represent the intermediates. Examples for platforms could be sugars, synthesis gas or lignin. The **secondary refining** comprise following processes to further refine the intermediates to semi-finished or end products. To fulfill the **requirements of a biorefinery**, the facilities have to comprise a primary and secondary refining step and need to disintegrate the individual biomass components. Further, the biorefinery needs to follow an integrated approach and has to produce not only one main product, but several. Following this classification, a sawmill or a biogas plant are no biorefineries for themselves. The sawmill lacks primary refining processes to disintegrate the wood into its components (cellulose, lignin, polyoses) and the biogas production plant just produces one product, which is power in form of electricity and heat (FNR, 2012). With this detailed classification, the definition of biorefineries stated in the German biorefinery roadmap differs slightly from the definition of the USDA and underlines the complexity of the topic. The description of the German bioeconomy council is in line with the classification described in the roadmap, as the holistic use of the material implements the processing of different products.

There are two approaches to describe the **development of a biorefinery**. The “bottom-up approach” relates to the extension of existing plants to utilize a side or waste stream for the production of further products. An example would be the extension of a pulp mill with a secondary refining facility to extract valuable components from the black liquor. On the other side is the “top-down approach”, which comprise completely new integrated biorefinery concepts without prior existing plants. The biorefinery roadmap points out five promising biorefinery concepts (FNR, 2012):

1. Sugar biorefinery and starch biorefinery
2. Vegetable and oil biorefinery and algal lipid biorefinery
3. Lignocellulosic (cellulose, hemicellulose and lignin) biorefinery and green biorefinery
4. Synthesis gas biorefinery
5. Biogas biorefinery

The review of Wenger and Stern (2019), which analyzed research articles about biorefineries (excluding the pulp and paper industry), showed that lignocellulosic crops, are the most abundant feedstock for biorefineries. Further, wood is the most targeted biomass within the group of lignocellulosic materials. Fuels as well as chemicals were found to be the main intermediate or final products of biorefinery processes. An included comparison with practical reports about existing biorefineries in Europe contrary showed a focus on biorefineries based on starch and sugar crops or oil and just a low proportion of lignocelluloses (wood) based ones. This controversial proportion of the topic in scientific literature and practical application is related to the varying and inhomogeneous characteristics of lignocellulosic materials, which are challenging for the digestion processes (Wenger & Stern, 2019). Wood as a material does not alone differ in its chemical composition and inhomogeneous structure, but the anisotropic properties as well as the hierarchic levels of the wood tissue are source of the materials complexity (Miletzky et al., 2020). However, lignocellulosic materials, such as wood, are biological composite materials in which the main compounds cellulose and lignin are

combined, which are the most abundant macromolecules on earth (Fengel & Wegener, 2003). Further, lignocellulosic resources are well available and do not compete to food resources, which enhances their potential as valuable raw materials for biorefineries (Karimi, 2015; Nanda et al., 2015).

Wood-based biorefineries require a process to disintegrate wood into its components. These processes can be for example chemical conversion processes like organosolv and kraft processes. Another option are thermochemical conversion processes like pyrolysis gasification or hydrothermal treatments. Lastly, there is the option to perform biological conversion processes like saccharification and fermentation. However, mostly a pretreatment like steam explosion is necessary prior to these conversion processes to increase the substrate accessibility (Berg & Guzmán, 2023).

2.4 Potential raw material sources for wood based biorefineries in Germany and conflicts of interests

To establish wood based biorefineries a secure supply with sufficient raw material is crucial and thus the available raw material sources need to be identified under consideration of possible conflicts of interests. In Germany, there are different potential future raw material sources conceivable, which originate from forestry, agriculture and industries.

2.4.1 German forestry as a raw material source

Forests cover about 32 % of Germanys land area, which is owned by private owners (48 %), municipalities (19 %) and by the federal countries (26 %) as well as the state (4 %). Roughly 45 % of the forested area is dominated by hardwoods the other half by softwoods, mainly spruce and pine (BMEL, 2021). In Germany, the commercial forests are managed in a way to meet economically, social and ecological needs at the same time. This includes for instance the individual harvesting of round wood, without clear cuts. This is beneficial for the continuous maintenance of a forest ecosystem and the simultaneous use of the area for relaxation and recovery of the public. Further, there are different concepts to support biodiversity within the forest for instance by leaving dead wood in the stands or diversification of the tree species portfolio, which improves the climate stability, too (BMEL, 2021).

There are various forest management concepts, described in literature. Additionally, in Germany, forestry is a matter of the individual federal countries, but overall these management strategies are very similar. Therefore, one management concept of Bavaria is presented in more detail as an example, to point out the different raw material streams, which originate from German forestry.

The **Bavarian management system** is structured by different management periods. The so called *qualification* period, describes the first 20 - 30 years including the forest stand initialization or rejuvenation. The period length is dependent on the species-specific growth speed during youth, which is quite high for instance for ash or chestnut and low for oak species. Main task during this period is a dense stand structure to promote the natural branch cleaning process. This qualification period is followed by a *dimensioning* period in which a specific amount of so called future trees (= Zukunftsbäume) are chosen, by different aspects like vitality, quality, fitness and distribution within the forest stand. These future trees are individually promoted in periodic small harvests (thinnings) to enable the development of vital trees with well developed treetops and high quality stem wood. For beech, oak and ash tender interventions are advised every five years. To keep a constant production level the rejuvenation of the forest stand is initiated stepwise already during the dimensioning phase (LWF, 2019). The trees, which grow in the area between the future trees, form a secondary stand, likely with a higher variability of tree species, which increase the biodiversity and generate additional economic value. Further, this secondary stand supports the maturing process of the future trees, by keeping a closed stand structure to promote a continuous growth rate (even year ring dimensions) and by shading the stems to prevent for example sunburn or the formation of second blooms (Bartsch et al., 2020).

Following this management strategy, tree breast height diameter (BHD) of 60 cm after 90 - 100 years are targeted for species like ash or maple. For beech, the rotation period is slightly longer between 100 - 110 years and a BHD of 60 - 70 cm is targeted. Oak species show the longest rotation periods up to 150 - 180 years with BHD of 70 - 80 cm. In contrast, for spruce the rotation period is just 60 - 80 years with a lower BHD of 45 cm (Bartsch et al., 2020).

With this background, two main raw material streams emerge from German forestry. The first one comprises the material from the **periodic thinnings** including different dimensions and

species during the dimensioning phase. The second raw material stream is the **final harvest** of the future trees at the end of the rotation period, targeting the production of quality round wood. The harvested material is classified into different assortments like stem wood, industrial and energy wood, based on the quality and dimensions of the stems or stem parts (DFWR & DHWR, 2015).

Regarding the future available round wood volume form German forests, the situation can be described as followed. The wood storage in German forests increased (2012 – 2017), but with a strong shift towards older tree stands in the age classes 61 – 120 years and > 120 years. For the age class 1 – 60 years there is strong decrease of 31 % for softwood and a slight increase of 5 % for hardwoods (Schmitz et al., 2022). Following the predictions of the WEHAM models, which cover the period 2013 – 2052, there will be a round wood potential of 77.7 mio m³ per year over all wood species (WEHAM, 2017). From this round wood volume, the proportion of oak (including all oak species) is just 8 %. However, the class of beech accounts to 29 % (22.4 mio m³/year), but just 25 % of it regards to beech as leading tree species in forest stands. Additionally, the class of beech includes other hardwood species (41 %) like ash or birch, except of oak. The biggest raw timber proportion accounts to spruce with 34,4 mio m³ per year. Comparing the raw timber potential of the species classes with the proportion of forested land, spruce takes 30 %, beech 34 % and oak 11 %. The missing species group is pine (including larch), which accounts to 26 % of the area and 15.2 mio m³ per year for the raw timber potential (WEHAM, 2017).

2.4.2 Short rotation coppices as raw material source

Next to the two main raw material streams originated directly from forestry, **short rotation coppice** could be another potential source of raw material for biorefineries. These are common agricultural areas, which are planted with fast growing tree species, mainly for the production of energy wood. These are managed at very **short rotation times** 2 – 20 years and show an average yield of 8 tons (atro) per ha and year. Suited species are poplar, willow or robinia, which are so called pioneer species. These species show a fast juvenile growth and a good root re-sprouting power after harvest (Landgraf et al., 2018; Matyssek et al., 2010; Veste et al., 2018). The ancient coppice management applied short rotation times (10 – 20 years) also for example for beech, oak or birch in the German Spessart region during the middle of the 19th century. Next to these species, also sweet chestnut was used for coppice systems (Konold, 2018). Next to a high productivity, the low maintenance of these systems is beneficial from ecological and economic perspective, due to less soil disturbance compared to annual crops and the management can be easily integrated into existing farming systems. Further, the cultivation of these crops can improve marginal land and compared to different waste streams the chemical composition is more homogenous (Dahmen et al., 2019).

2.4.3 Other raw material sources and conflicts of interests

In Germany (2020) the amount of wood from harvests, imports and storage decrease was 91.8 mio m³ with a hardwood proportion of 20 %. In numbers that means 71.9 mio m³ of softwood, which was mainly aligned to material utilization (90 %) and about 19.9 Mio m³ of hardwood. However, just 10 % of the hardwood proportion was used for material utilization, but 90 % were used for energy production (Schmitz et al., 2022). There are three main industry sectors the saw mill industry (28.5 %) the wood composite industry (12.3 %) and the pulp and paper industry with 7.7 %, which share the biggest proportion of the material utilization (FNR, 2018).

The volume of **sawn timber** in 2015 was 36.0 mio fm, with a proportion of 93.5 % accounting to softwood and 6.5 % to hardwood stems. The volume of sawn softwood decreased from 2010 to 2015 with -3.8 %, in contrast the volume kept nearly constant for hardwoods (Mantau et al., 2018).

The wood volume for the **pulp and chemical pulp industry** was 10.0 mio fm in 2015. The proportion of industrial wood from softwood was 53.4 % and saw mill residues accounted to 38,3 %. Industrial wood from hardwoods were solely used for the chemical pulp production (8.3 %) (Mantau et al., 2018).

The **wood composite industry** in 2015 used 15.8 Mio fm of pulp wood. A proportion of 39.3 % were accounted to saw mill residues and 31.2 % to industrial wood from softwood (debarked). Industrial wood from hardwood (debarked) shared a proportion of 13.7 % and recovered wood about 11.7 %. Other residual woods from industrial processes accounted to 1.9 % and bark had a proportion of 2.3 % (Mantau et al., 2018).

For **private firing**, the wood volume was 27.6 Mio fm in 2014. Log wood from forests had the largest share with 71.6 %, but also used wood and wood pellets hold a share of 4.6 % and 8.3 %. The residual proportions are shared between landscape wood residues, log wood from gardening, sawn timber residues, wood chips, wood briquettes and kindling wood (Mantau et al., 2018). For **large-scale biomass combustion** plants 13.3 Mio tons of wood were used in 2016. The recovered wood shared with 48.6 % the biggest proportion, followed by forest residues with 12.3 % and landscape wood residues with 13.2 %. The residuals from wood processing accounted to 4.9 % and wood residues from sawn timber production about 5.9 %. Additionally, 8.5 % were accounted to bark and 4.4 % to others (Mantau et al., 2018). For **combustion plants** smaller than 1 MW the raw material portfolio changes again with main proportion shared by forestry residues with 31.4 %, residuals from saw timber production with 18.4 %. The residual proportion accounts to other wood residues (numbers from 2016) (Mantau et al., 2018).

These numbers clearly show the low proportion of hardwoods in terms of material utilization. With this breakdown of the different assortments used in the wood industries and for energy production, further potential raw material streams for lignocellulosic biorefineries can be identified. These are the different kinds of wood residues from the wood industry like saw mill residues as well as the fraction of recovered wood. Regarding the assortment of **recovered wood**, it has to be considered that this fraction is likely, to be contaminated with impurities, due to former use in constructions or as part of composites. Additionally, the wood used in constructions often was treated with coatings or wood preservatives, which contained halogenic substances or heavy metals, which are nowadays no longer approved (Schmitz et al., 2023). The utilization and the disposal of recovered wood is regulated in German legislation within the so called “Altholzverordnung” (BMJ & BfJ, 2002). Therefore, the assortments of recovered wood potentially are challenging for biorefinery processes due to inhomogeneity and unwanted contamination of processed products, which could be an environmental thread or harmful for human health.

The potential raw material source of **industrial wood residues** like sawdust is an interesting option as depending of the origin, these materials are homogenous and without impurities. However, this source is already well included in the current wood market and an additional actor like a lignocellulosic biorefinery would increase the distribution competition between the different actors.

The presented breakdown of the assortments further points out different **conflicts of interests**, which will be shortly examined in the following. There is a high demand of high quality softwood for various applications like constructions and the numbers clearly show that softwood dominate the different material utilization routes (FNR, 2018). The current adaptation

of German forests towards climate stable forest stands by increasing the hardwood volume and species diversity stands against this market demand (Miletzky et al., 2020). A first reaction of the processing industries would be to compensate missing softwood capacities by imports. However, the lack of profitable demand markets for the arising hardwood volumes, would hamper the motivation of forest owners to actively manage their forest, which would be disadvantageous for a future stable forestry and thus to secure raw material supply (Miletzky et al., 2020). The reduce of the softwood demand by innovative product application as well as substitution of currently softwood based products by hardwood based products would be more in line with circularity strategies and thus with a sustainable development. Further, an adapted forest management has not to exclude softwood species completely, but needs to find a well suited balance between the tree species. Due to the high proportions of softwood for material utilization, the industrial wood residues, which were identified as potential raw material source for biorefineries, are mainly from softwoods and thus will likely needed to cover the market demand.

Another conflict of interests is addressed by the German bioeconomy strategy and the recommendations for action of the German bioeconomy council, as these promote the enhancement of material use over energy use (BÖR, 2023). From the perspective of a cascading use of resources, an energetic use of wood is not profitable, as this volume could also be used for material utilization routes. Additionally, the production of fine aerosols from small firing plants in private households, which are currently very popular, are an environmental thread and possibly outrage the benefits of a renewable energy source. However, on the other side the stable and profitable prices for energy wood are the motivation for many forest owners to actively manage their forest stands as they can sell the assortments from thinnings for instance as raw material to produce pellets. If this sales market is omitted the same disadvantageous development as described for the softwood – hardwood conflict of interest is likely (Miletzky et al., 2020).

On the one side, the implementation of alternative utilization routes for hardwoods thus seem to be the best option to maintain forest management, which targets actively the production of wood. On the other side, this enables an increase in the material utilization proportion of hardwoods. This leads to the conclusion that the prioritized raw material sources for lignocellulosic biorefineries likely will be hardwood originated from thinnings or short rotation coppices as these are currently mainly used for energy production, due to low BHDs or low quality.

With this focus on energy wood assortments another topic arises, the valorization of bark. Wood assortments commonly used for combustion do not include debarking, but use the material as an additional energy rich fuel (Teischinger et al., 2023). Additionally, wood from thinnings or short rotation coppices often have a low BHD, which result in a higher proportion of bark based on the total volume (Rössler, 2008). Further, most of the harvested trees in thinnings will have a higher amount of branches compared to the future trees. The top of a tree as well as branches show the highest bark proportions (20 - 30 %). The bark proportion further depends on the growing conditions and the trees age (Fengel & Wegener, 2003; Wagenführ, 1984).

2.5 Possible product portfolios of wood-based biorefineries

The separation of the individual biomass components is an obligatory characteristic of lignocellulosic biorefineries (FNR, 2012). As a result, the product portfolio is based on the main components, in the case of wood, on cellulose, hemicelluloses and lignin as well as wood extractives. The latter one contain different substance classes, which are soluble in different (organic) solvents and are not assigned to cellulose, lignin or hemicelluloses. Due to the

hierarchic structure of wood, it is possible to use these components as individual products or to further break down the macromolecular structures and use the monomeric molecules or smaller polymers directly or as starting material for further syntheses (Berg & Guzmán, 2023). For a more detailed chemical description of the individual wood components, please refer to chapter 2.7. The spectrum of possible products derived from lignocellulosic biomass is tremendous and there is a high amount of research in the different fields, thus this chapter cannot comprise all options, but gives an overview on some aspects.

2.5.1 Cellulose derived products

There are several well established utilization paths for the cellulose, next to classical paper production. These are for instance the production of **nanofibrillated cellulose** also called microfibrillated cellulose (MFC), with cellulose fibers in the nanoscale range of 1-100 nm. MFCs show good material properties, like a high specific surface area and good abundancy of hydroxyl groups or good gas-barrier properties next to others. They are suitable for applications in aerogels, hydrogels or in combination with other media in the form of nanocomposites. In contrast to MFC, which is produced by high-pressure homogenization, **microcrystalline cellulose** (MCC) is produced by hydrolysis of cellulose with mineral acids. These mainly crystalline structures are currently used in plastic or cement production. Further, there are some applications in the food, cosmetic and pharmaceutical industries (Berg & Guzmán, 2023).

Another well established group of cellulose products are **cellulose derivatives**, mainly cellulose esters and cellulose ethers. The first one is produced by esterification of cellulose with organic acids like propionic acid and acetic acid or by the reactions with anhydrides. A prominent example for cellulose esters is for instance cellulose acetate. This substance group is widely used to produce different kinds of fibers, films or coatings. Further cellulose esters are used to produce plastics and different additives. In contrast, cellulose ethers are produced by a reaction in alkaline medium and there are various varieties of cellulose ethers available, like methylcellulose or hydroxypropylcellulose. For instance, by different modifications cellulose ethers can be used as surfactants. **Cellulose regenerates** are highly requested for the production of fibers and films, which are biodegradable and biocompatible. Principally, the cellulose fiber is first solved in a solvent and then regenerated within a coagulant with adapted properties. Further, these products have a good thermos and chemical stability. The most prominent example is the viscose fiber (Berg & Guzmán, 2023). Especially in the cellulose fiber section new technologies and concepts become more and more important and start to reach an economical feasible level, examples would be Ioncell® or Spinnova® (Verkerk et al., 2022).

Further, by breaking the cellulose structures down to monomeric glucose sugars (see chapter 2.7.1) followed by fermentation the glucose can be converted to **ethanol** for fuels or as platform chemical for the production of butadiene and ethylene. The first one is a promising intermediate for the polymer industry, especially for the synthetic rubber production and biobased ethylene can substitute fossil based ethylene within the polyethylene production (Cherubini & Strømman, 2011; Gómez Millán et al., 2019; Zamani, 2015).

2.5.2 Products derived from hemicelluloses

Next to cellulose the hemicelluloses form an interesting substance class. However, in contrast to cellulose the hemicelluloses show a higher variety in case of the monomeric sugars (pentoses and hexoses) and the macromolecular structure (also see chapter 2.7.2), which makes the utilization of these substances more challenging. Like glucose from cellulose, the

hemicellulose sugars are potentially suitable to produce ethanol by fermentation. However, there are less microorganisms available suitable to convert pentoses or a mixture from pentoses and hexoses. The **polymeric sugars**, for instance xylooligosaccharides are used in medicinal applications to support the growth of probiotic bacteria in the colon. Additional, xylans have some potential for the application in biodegradable food packaging due to film forming properties and there are some approaches to use xylans for the production of surfactants. Another interesting group within the hemicelluloses are the xyloglucans. In plants these substances crosslink cellulose fibrils within the primary cell wall and are responsible for the storage of carbohydrates. Xyloglucans have a wide field of applications in the food, pharmaceutical, paper, biomedicine or cosmetic industries due to highly requested properties like gelling, stabilizing as well as antiviral and antioxidant characteristics (Berg & Guzmán, 2023). The spectrum of applications for hemicelluloses becomes even wider, if the focus is set on the **individual monomeric sugars**, their derivatives and synthesis products. For example for woody biomass the main monomers are D-xylose and L-arabinose as pentoses (C5 sugars) and D-mannose, D-galactose and to a small extend L-rhamnose, as hexoses (C6 sugars). These monomeric sugars or their derivatives are highly requested for example for pharmaceutical applications or as alternative sweetener. D-mannose for instance, can bond to specific bacteria and remove them from the digestion system or can be simply used as alternative sweetener. Xylitol the sugar alcohol derived from xylose is highly used as an alternative sweetener like D-mannose. Further, xylose has a lower calorific value and is less harmful for teethes than sucrose and does not affect the insulin metabolism (Fengel & Wegener, 2003; Mäki-Arvela et al., 2011)

Other valuable products from the **fermentation of pentoses** (C5 sugars) are 2,3-butanediol and lactic acid. The first one is applied in the production of inks, pharmaceuticals or food additives (Berg & Guzmán, 2023). The food industry purchases 70 % of the produced lactic acid, which is a potential building block for biodegradable plastics, like polylactic acid (PLA). Succinic acid is a fermentation product of biomass and a promising intermediate due to its two carboxylic groups and can possible be used for the production of resins, foams or surfactants, next to others (Gómez Millán et al., 2019).

There is also the group of so called **sugar degradation products** originated from C_5 or C_6 sugars like furfural and hydroxymethylfurfural (HMF). These substances are often seen negatively due to their inhibitory effect on enzymes or the fermentation process (Talebnia, 2015), but actually are highly valuable products. Furfural is a well suited and applied solvent for the extraction of aromatic substances, next to others (Berg & Guzmán, 2023). Additionally, furfural is a good platform for the synthesis of further substances, like furfuryl alcohol. There are more than 80 different valuable derivatives, which can be synthesized from furfural. The synthesis of furfural to furfuryl alcohol accounts to 60 % of the total furfural market. Furfuryl alcohol is used for example in the food industry, to produces foundry resins (Gómez Millán et al., 2019; Li et al., 2016) or for wood modification like in Kebony® wood. Additionally, furfural can be converted to furoic acid, maleic acid, succinic acid and multiple more (Berg & Guzmán, 2023). HMF can for instance be converted into levulinic acid, which is a promising platform chemical due to its carboxyl and carbonyl functionalities. Possible applications would be in food or animal feed as well as polymer industries. Further, 2,5-furandicarboxylic acid (FDCA) can be synthesized from HMF. FDCA is a very valuable substance as it can be used in the polyester production instead of the fossil-based substances terephthalic acid and polyethylene terephthalate (Gómez Millán et al., 2019). FDCA can be further converted to succinic acid or furan derivatives with potential for the production of polymers and fuels (Berg & Guzmán, 2023).

2.5.3 Products derived from lignin

Next to cellulose and hemicelluloses, lignin is an abundant component in lignocellulosic biomass, especially in woody material. Lignin is a natural source of aromatic compounds, but the high complexity of its chemistry regarding isolation and modification often hampers the implementation of big-scale material utilization routes. Despite these challenges, there are already some fields of application and high potentials for further operating ranges. Commercial applications are present for instance in the field of synthetic resins and adhesives. For example for the production of polyurethanes and the partly substitution of fossil based phenols by phenols originated from lignin in phenol-formaldehyde and urea-formaldehyde resins. There is also research in adhesive formulation combining lignin with other substances like isocyanates or tannins. Another field of application in industrial scale are plastics, mainly thermoplastics. Additional, there is research to use lignin for the production of carbon fibers or in electrochemical applications as well as in the agricultural sector as medium for the controlled release of fertilizers.(Berg & Guzmán, 2023; Miletzky et al., 2020; Verkerk et al., 2022).

2.5.4 Products derived from extractives and humins

Wood extractives share just a small proportion of wood or other lignocellulosic material, but have high potentials for special valorization for example as fine chemicals. There is a tremendous variety of individual substances accounted to the group of extractives, which are partly already used or under research. The low proportion of extractives reduces the economic feasibility in some cases. However, due to the good solubility of extractives in different solvents and positive effects of the extraction in following biorefinery processes, the utilization becomes more and more interesting (Berg & Guzmán, 2023).

The substance class of **tannins** is present in many plants and different parts of the plants like fruits, but also the bark. In terms of wood-based biorefineries, tannins thus would be also an interesting product, if bark is processed. The various monomers and polymers of tannins show a complex chemistry and within the plants, tannins are formed in the secondary metabolism. For example, the heartwood of oak and chestnut trees contains a high proportion of tannins, which are responsible for the high natural durability of the wood (Berg & Guzmán, 2023; Feng et al., 2013; Fengel & Wegener, 2003). Tannins can be grouped into condensed and hydrolyzed tannins (please refer to chapter 2.7.4 for chemical details), but both groups are well known to precipitate proteins. The application of these substances for leather tannin is performed since ancient times, meanwhile the use of chrome salts is economical more feasible, but due to the current developments and the toxicity of these salts, plant-based tanning becomes more and more interesting again. Other field of applications feasible for tannins are adhesives for example for the production of particle boards or plywood as well as for different pharmaceutical applications. In the adhesive production, hydrolyzed tannins probably could be used to substitute fossil-based phenols in adhesive formulations, similar to lignin, and condensed tannins can cross link with different agents for instance formaldehyde to produce sole binders. The molecular structure of tannins has a strong influence on the performance and is thus a critical factor. These developments are due to their capability to form bonds with polysaccharides and lignin (Berg & Guzmán, 2023; Mai & Zhang, 2023). Regarding pharmaceutical applications, tannins have anti-inflammatory, antimicrobial or antioxidant properties. However, the challenge is to produce these substances in sufficient amount and purity (Sticher et al., 2015).

Another group of interesting extractives are **terpenes** and **terpenoids**, which are also a very versatile substance group and most abundant in softwoods. These substances are actively linked to the trees metabolism and are for example responsible for defense, flavors or sometimes colors of plants. Therefore, the field of applications is quite wide and includes

fragrances, flavorings, but also pharmaceutical applications, next to others. Monoterpenes and sesquiterpenes are volatile at room temperature, with α- or ß-pinene as prominent examples, which are linked to the typical odors of pine species. The substance betulin in birch is a triterpene and has high pharmaceutical value, as it has anticancer properties and can be used as starting substance for the production of anti-HIV drugs. In the case of **waxes** or **aliphatic compounds**, fatty acids are used for instance to produce emulsifiers, adhesives or surfactants. Parallel to that fatty alcohols can also be used to produce ionic surfactants or are for example used on paper products. Pinosylvin in pine species or resveratrol for instance in eucalyptus are some examples for **stilbenes**, which are another group of extractives with highly interesting pharmaceutical applications due to cell death activation or anti-inflammatory properties. Similar to stilbenes the substance groups of **flavonoids** and **lignans** are very interesting for pharmaceutical applications (Berg & Guzmán, 2023; Fengel & Wegener, 2003). Further, lignans play a role in the seasoning of wine (Cretin et al., 2015), which opens up a field of application in the beverage industry.

Next to the different products derived from the different lignocellulosic components, there is ongoing research on the valorization of new substances evolved from the reaction processes in lignocellulose biorefineries, for instance the substance group of **humins**. Current research tests these side streams for instance for their suitability to form thermoset polymers (Cantarutti et al., 2020; Pin et al., 2014).

B. Wood and bark– anatomical and chemical properties

2.6 Anatomical and biological wood features relevant for the material properties

The wood tissue is a hierarchical complex natural composite with various variation between different tree species. Some of these species-specific differences are related to the wood formation as well as the cells, their distribution, functionality, and dimensional properties.

2.6.1 Wood formation

A meristematic tissue called vascular cambium is located as a thin cell layer between the wood and the bark. Through the formation of xylem cells to the inside and phloem cells to the outside the vascular cambium builds up the wood and bark tissue. The xylem is responsible for the mechanical strength of the tree stem. The active region of the xylem, directly next to the cambium is additionally assigned to the conduction of assimilates. The phloem is part of the bark also assigned to assimilate transportation. (Schmitt et al., 2023). Details about bark are presented in chapter 2.8

The newly formed xylem cells first undergo different development or differentiation processes. The first step is **cell expansion**, increasing the cell volume until the cell reaches its full size. In this phase, the cells are solely boarded by the primary cell wall, which is flexible enough to adapt to the extension forces, due to the content of pectic polysaccharides, hemicelluloses and structural proteins. The next differentiation step is the **formation** of the **secondary wall**, which is sectioned into three sublayers (S1, S2 and S3). The S1 layer is located directly next to the primary cell wall, followed by the S2 and S3 layer towards the cell lumen. In most cases, a wart layer towards the cell lumen boards the secondary cell wall (S1-S3). Within the secondary cell wall, which contains lignin, cellulose and hemicelluloses, the S2 layer is most important for the mechanical properties of the wood and contains the biggest cellulose proportion. The third differentiation phase is the **lignification**, starting at the primary cell wall and the middle lamella, which is the region, where the different cells touch each other. During the lignification phase, specific cell types, like hardwood vessels (see chapter 2.6.2) undergo **programmed cell death** to become completely functional. This is especially important for conducting cells, to enable the most effective water transport (Fengel & Wegener, 2003; Schmitt et al., 2023).

The trees metabolism reacts on the periodic changes of growing conditions and thus form early wood and late wood zones, which together form one year ring. Early wood aligns to the proportion of the year ring, which is formed during the beginning of the growing season with strong conducting properties, followed by a late wood zone, which is formed afterwards and is mainly attributed to strengthening. Main difference between early and late wood cells is the thickness of the S2 layer, which comprise around 60 % of the tissue volume in early wood and 80 % in late wood (Fengel & Wegener, 2003; Schmitt et al., 2023).

Under specific environmental strains, the tree forms specific reaction wood like compression or tensile wood, which differ chemically and physically from the normal wood tissue. Compression wood shows higher lignin contents and there is no S3 cell wall layer and the S2 layer is modified. This type of reaction wood is typical for softwoods. Tensile wood is common for hardwoods and contains higher proportions of cellulose within an additional cell wall layer the G-layer (Fengel & Wegener, 2003).

The part of the xylem directly next to the cambium forms the **sapwood**, which contains living fiber and parenchyma cells. The parenchyma cells actively participate in the trees metabolism. Therefore, these cells not only contain cellulose, hemicelluloses and lignin, but additional components like fatty acids or starch, which are linked to the trees metabolism. These substances are referred to as primary extractives. If the tree forms **heartwood**, the parenchyma cells of the inner year rings also undergo cell death and thus do no longer participate in the trees active metabolism and primary extractives are degraded and secondary extractives are stored, mainly phenolic compounds, which are responsible for the color change of the heartwood region for some species. The presence of heartwood depends on the tree species and varies strongly in its appearance and properties, but additionally depends on other factors like environmental conditions, age or injuries (Fengel & Wegener, 2003; Grosser, 1977; Schmitt et al., 2023).

There are different heartwood formation types. Species with **mandatory heartwood** formation like oak and chestnut or pine and larch show a decrease in wood moisture and a high content of extracts in the heartwood region, which increases the natural durability of the heartwood. Typical for the heartwood formation is the sealing of the vessels, by organic material or tyloses, both originated from neighboring parenchyma cells through pits. Tyloses are little protrusions, common in species like oak, chestnut or robinia. Interestingly, the vessels of red oak *(Quercus rubra)* show no or just very low presence of tyloses. Like the high extract content, the sealing of the vessels should enhance the wood resistance against fungal and insect attacks and thus increase the durability. The high extract content is due to the storage of secondary wood extractives in the cell walls. For oak and chestnut, these are mainly tannins (Fengel & Wegener, 2003; Grosser, 1977; Schmitt et al., 2023).

Contrary, to oak or chestnut species, some species like beech *(Fagus sylvatica*) and ash *(Fraxinus excelsior)* form a **heartwood,** which is **not differentiated by color.** Nevertheless, the function and activity of the cells are affected as well as the moisture content, which decreases in the heartwood region. However, some species can form a **facultative heartwood**, which results in distinctly different properties of these wood proportions. These facultative heartwoods show less uniform shaping and a markedly reduced durability compared to mandatory heartwood. The formation of false heartwood is triggered by reactions of the tree to special environmental conditions, like injuries or age effects. Beech *(Fagus sylvatica*) and ash *(Fraxinus excelsior)* are typical examples for the optional facultative heartwood formation. Lastly, there is the group of **sapwood-trees**, which includes for instance birch or hornbeam. The outer and inner part of the stem does not differ in moisture or color and the parenchyma cells stay alive in the xylem from the cambium to the pith (Fengel & Wegener, 2003; Grosser, 1977; Schmitt et al., 2023; Wagenführ & Scheiber, 1974).

2.6.2 Different cell types, their characteristics and role in the wood tissue

The wood tissue contains different cell types, which fit the need of conducting water and assimilates and strengthening the wood tissue. Additionally, the tissue needs to store reserve and primary metabolic substances. Regarding the structure, strong differences between softwoods and hardwoods arise, as the latter ones are evolutionary younger and thus show a higher cell function specialization than softwoods (Schmitt et al., 2023). Figure 1 presents graphic display of different wood cell types and their position in the tree stem to assist the following explanations.

Softwoods comprise mainly **tracheids** and **parenchyma cells**, which have functions of strengthening, conducting as well as storing and are supplemented with specialized parenchyma cells also referred to as epithelial cells, which contain resins or other secreting substances. The different cells are connected by different types of pits. A characteristic pit type

for softwood species like pine, larch, fir or spruce are the so called bordered pits, which can irreversibly close the passage, if there is for instance a change in pressure.

In contrast, hardwoods contain **libriform fibers** and **fiber tracheids**, which are long cells, mostly with thick cell walls. These cells are responsible for the tissue strengthening. Additionally there are **parenchyma cells** for storing and **vessels** for the conducting part. Especially the vessels are very unique for hardwoods as these cells are connected at the end by wide passages, also referred to as vessel perforation plates, which enables high volume transports of water and assimilates. These vessel "pipes" (multiple vessel cells, connected by perforation plates) can reach a length of 0.8 – 2.0 m or 5 – 18 m for beech *(Fagus sylvatica)* or oak *(Quercus robur).* Tracheids, fibers and vessels are orientated axial and parenchyma cells can be orientated axial or radial, for instance in case of wood rays. In some coniferous species tracheids flank the wood ray parenchyma cells and are responsible for water conduction in radial direction (Fengel & Wegener, 2003; Grosser, 1977; Schmitt et al., 2023).

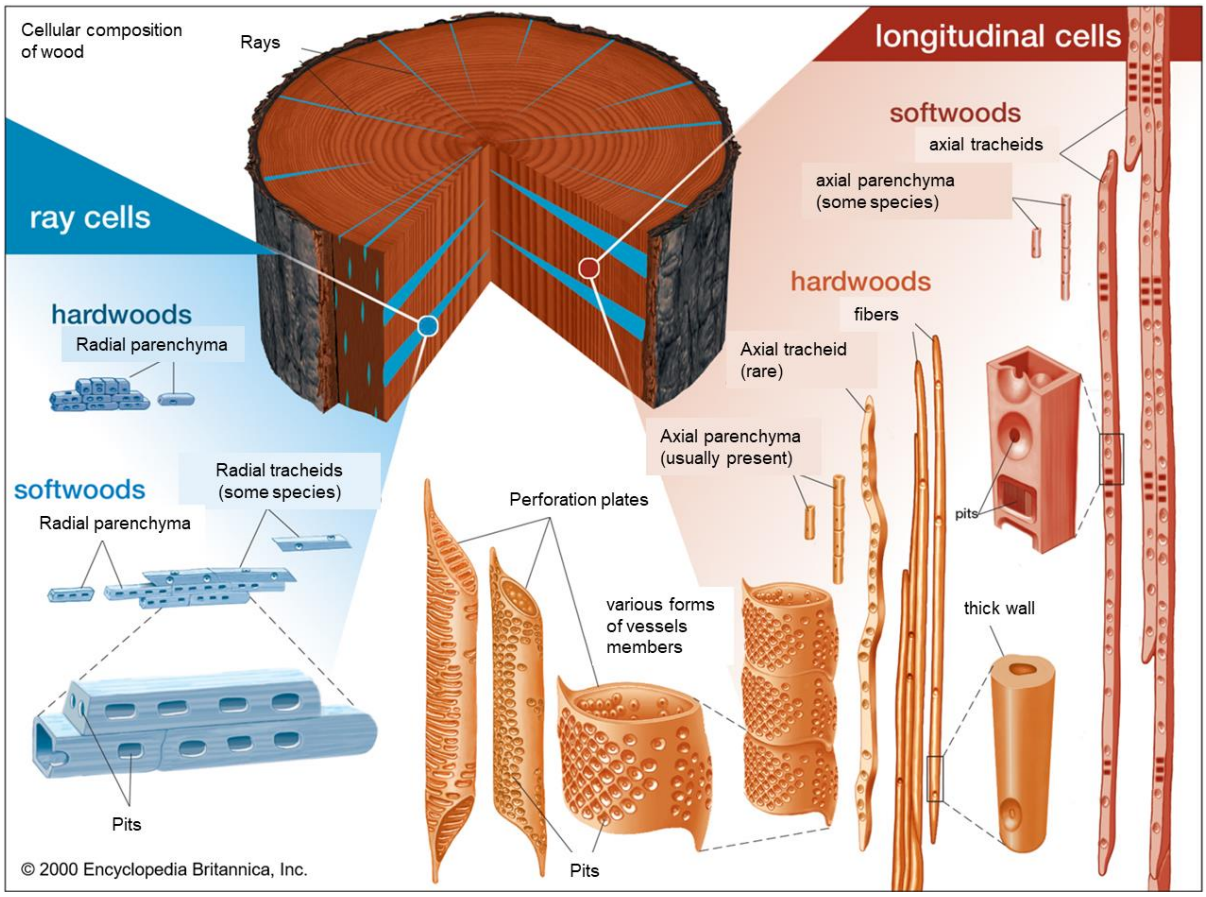


Figure 1: Cell types in hardwood and softwood, graph from the article of Encyclopedia Britannica (2023); labelling enlarged.

The arrangement of the vessels within a year ring is one macro-molecular feature, which is characteristic for specific tree species. Different oak species for example account to the group of **ring porous** species with wide vessels, which are located circular in the early wood zone and smaller vessels in the late wood zone of the year ring (Fengel & Wegener, 2003). The vessels have a diameter of 15 - 350 µm for early wood and 30 - 140 µm for late wood vessels, with a total proportion ~ 39.4 vol% within the wood tissue. In contrast, beech accounts to the **diffuse porous** species, where the vessels are general smaller and distributed evenly across the early- and late- wood zone with diameters of 8 - 85 µm and a proportion of ~ 39.5 vol%

(Wagenführ & Scheiber, 1974). Because of the diffuse pore distribution, the raw density does not depend on the wide of the trees year rings (= ratio early wood / late wood section) as it is the case for ring porous species. Additionally, the mechanical properties are less dependent on the climatic conditions in the vegetation period. The wood tissue of ash *(Fraxinus excelsior)* and chestnut *(Castanea sativa)* show both a ring porous distribution of the vessels over the year ring section similar to oak. Additionally, there is the type of semi-ring porous wood, which show a higher amount of vessels or a slightly bigger vessel diameter within the early wood (Fengel & Wegener, 2003; Grosser, 1977; Wagenführ & Scheiber, 1974).

For the radial transportation of assimilates parenchyma cells form **radial wood rays**, which can differ in their dimensions. For instance, there are well visible wood rays in cross sections of oak *(Quercus robur/petraea)* and beech *(Fagus sylvatica).* For oak, the proportions are dependent on the wide of the year ring with 16.2 % for narrow and 29.3 % for wide year rings. For beech, there is a high proportion of wood rays with 27.0 %, which is especially known to enable a good liquid transport not just in tangential direction through the vessels, but also in radial direction (Fengel & Wegener, 2003; Grosser, 1977). For the **fibers**, which comprise to the tissue strengthening in hardwoods the length is the crucial factor. Beech *(Fagus sylvatica)* contains very short fibers with 600 - 1300 µm, the fibers of ash *(Fraxinus excelsior)*, oak *(Quercus robur/petraea)* and chestnut *(Castanea sativa)* are longer (Wagenführ & Scheiber, 1974). Table 1 contains values of the proportions and properties of the different cell types as well as values for the raw density as basic information about the species. The macro- and microscopic properties of wood are partly species-specific. The knowledge about these characteristics is important to evaluate the potential influence during processing for instance in case of the permeability of the material, which could be important for cooking or steaming processes.

Table 1: Vessel and fiber proportions in hardwoods; (no differentiation between Quercus robur an Q. petraea) (Wagenführ & Scheiber, 1974)

	Units	**European beech** *(Fagus sylvatica)*	**Common ash** *(Fraxinus excelsior)*	**English oak** *(Quercus robur)*	**Sessil oak** *(Quercus petraea)*	**Sweet chestnut** *(Castanea sativa)*
Raw density*	[kg/m³]	~ 720	~ 720	~ 690		~ 620
Vessels						
Type		diffuse	ring	ring		ring
Ø early wood	[µm]	8 - 85	60 - 350	15 - 350		150 - 300
Ø late wood	[µm]		15 - 130	30 - 140		36 - 125
Proportion	[vol %]	~ 40	~ 12	~ 39		~ 26
Fibers						
Length	[µm]	600 - 1300	150 - 1600	1230 – 1740		600 – 1570
Proportion	[vol %]	~ 40	~ 62	~ 51		~ 57

* based on 12-15% wood moisture

2.7 Chemical composition of wood

2.7.1 Cellulose

With 10^{11} – 10^{12} tons of cellulose produced per year by photosynthesis, cellulose is a highly abundant biopolymer and thus a valuable resource for various applications. Cellulose in hardwoods accounts for 40 – 50 w % and is an important structural part of the plant, like hemicelluloses or lignin and is responsible for the tensile strength of the wood. In general, cellulose is a linear polymer, solely containing anhydrous glucose molecules, which are connected by **ß-(1 → 4)-glycosidic linkages**. Like wood, cellulose has a hierarchical structure of its own. The individual chains of anhydrous glucose molecules represent the **molecular** and **macromolecular level**. This level is also used for the characterization of cellulose for example by the degree of polymerization (DP) or the molecular weight as values to describe the chain length. For example, aspen wood has a degree of polymerization of around 10.300 and birch wood of around 9.400. The **supermolecular level** is represented by the elementary fibrils with a diameter of ~3 nm and microfibrils or macrofibrils with diameters between 10 - 25 nm, which form through the aggregation of individual cellulose chains. Those fibrils then agglomerate further to form spiral structures in the different cell wall layers to form the final cellulose fiber **(morphological level)**. The agglomeration of the individual cellulose chains and fibrils of the different hierarchic levels and the linear structure of the polymer is possible due to the hydroxyl groups and oxygen of the linked ß-D-anhydroglucopyranose units. This enables multiple **intra- and inter-molecular hydrogen bonding**, which stabilizes the polymer chemically and mechanically (Fengel & Wegener, 2003; Mai & Zhang, 2023).

In the elemental fibrils of the supramolecular level, the cellulose chains can be arranged in a **crystalline** or **amorphous** manner, commonly with ~70 % crystalline and ~30 % amorphous structures. These structural aspects are important characteristics for different utilization routes like biological conversion. The crystalline regions are highly structured and thus more resistant to disintegration compared to amorphous regions due to a reduced reactivity towards solvents and a reduced reaction surface. The cellulose type derived from biosynthesis and thus most important for biological conversion processes is classified as cellulose I, which is mainly present as monocyclic crystal structure ($I_ß$) at least in higher plants, like wood. Cellulose I is one of the four different crystalline polymorphs of cellulose. Cellulose II-IV are the products of different cellulose modifications and important for different utilization routes of cellulose (Fengel & Wegener, 2003; Gupta, 2016; Mai & Zhang, 2023; Shafiei et al., 2015).

2.7.2 Hemicelluloses and pectins

Hemicelluloses (polyoses) are complex polymers build out of different hexoses (C_6 sugar), pentoses (C_5 sugar), uronic acids or deoxy-hexoses. The main hemicellulose sugars in wood are D-xylose (xyl), D-glucose (glc), D-galactose (gal), D-mannose, L-arabinose (ara), L-rhamnose (rha) and D-glucoronic acid. Hemicelluloses form shorter molecular chains (DP 100-200) and includes branching. In comparison, cellulose has solely a long linear structure. Due to the more irregular structure and lower chain lengths, hemicelluloses are less chemically and thermal stable than cellulose, which is challenging for a simultaneous utilization of both components. Within the cells, hemicelluloses are mainly present in the secondary cell wall and support the cell wall structure due to forming an interface between the cellulose fibrils and lignin (Ek et al., 2007; Fengel & Wegener, 2003; Mai & Zhang, 2023).

In relation to the main backbone-sugar molecule, the group of hemicelluloses comprise xylans, mannans, xyloglucans and ß-glucans. The tree species differ in their hemicellulose composition and especially softwoods contain much more mannose and galactose dominant structures than hardwoods. The most important hemicelluloses of hardwoods are the **xylans**.

For instance in hardwoods glucuronoxylans share a proportion of 15 – 30 % (wt) of dry mass including D-xylose as backbone units (ß-(1→4) linked). Additionally, units of 4-O-methylglucoronic acids form the branches by α-(1→2) glycosidic linkages and O-acetyl groups substitute the free hydroxyl groups. The respective molar ratios of these units is 10, 1 and 7 and the DP of glucuronoxylans is around 200. Glucomannans are also present in hardwoods with 2 – 5 % (wt) of dry mass. This hemicellulose contains D-mannose and D-glucose units, both ß-(1→4) linked with molar ratios of 1 – 2 and 1 respectively. Glucomannans have a DP of 200 like the glucuronoxylans (Fengel & Wegener, 2003; Mai & Zhang, 2023).

Unlike hemicelluloses, **pectins** just share a small proportion in wood, but are most abundant in the middle lamella. Main function of pectins for instance moisture balancing or cementing the cellulose fibers. The substance group of pectins is very versatile and complex, with D-galacturonic acid as most abundant constituent, but comprising around 17 different monosaccharides combined by 20 different linkage types (Ek et al., 2007; Mai & Zhang, 2023).

2.7.3 Lignin

Lignin is a natural, amorphous polyphenolic molecule with an annual production of around 5 – 6 x 10^8 tons. Lignin amounts to 20 – 40 w% of the wood structure, but there are differences between softwoods and hardwoods and individual tree species. Hardwoods contain generally less lignin with 17 – 25 % than softwoods with 27 – 35 %. The lignin increases the stiffness of the cell walls and due to the hydrophobic properties the sorption of water is reduced. The high proportion of lignin within the middle lamella further stabilizes the wood tissue, as lignin is not hydrolysable and due to the reduced water permeability, the tissue becomes more resistant against various pathogenic degradation agents, except of specialized individuals (Mai & Zhang, 2023).

The basic building blocks of lignin are p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The formation of macromolecular structures out of these building blocks works over dehydrogenative polymerization with the most prominent linkage being the ß – O – 4 linkage, accounting to 50 – 60 % of all linkages. Other possible linkages are ß – ß or ß – 5, next to others. However, the proportion of the linkages varies between softwoods and hardwoods. For example, the ß – O – 4 linkage has a higher proportion in hardwoods with 50 – 70 % compared to 35 – 60 % in softwoods. In contrast, the ß – 5 linkages account to 4 – 9 % in hardwoods, but 9 – 12 % in softwoods and the ß – ß linkages have similar proportions (Fengel & Wegener, 2003; Windeisen & Wegener, 2012)

Within the three-dimensional lignin structure, the original building blocks are detectable by their aromatic-ring structure including their functional groups. The building block p-coumaryl alcohol is thus referred to as p-**h**ydroxyphenyl units, the coniferyl alcohol forms the **g**uaiacyl units and the **s**inapyl alcohol forms the syringyl units. The proportion of the different building blocks within the lignin structure differs between the plant species. In grasses all three building blocks are present and thus from HGS lignins. Softwood lignin mainly contains **g**uaiacyl units and is thus referred to as **G**-lignin. Hardwood lignin has high proportions of **g**uaiacyl and **s**yringyl units with 25 – 60 % and 45 – 75 % respectively and is thus classified as **SG**-lignin. For example, the lignin composition of beech was reported with 56 % G-lignin and 40 % S-lignin as well as 4 % H-lignin, as wood also contains a small proportion of this lignin type. According to their origin (coniferyl alcohol and sinapyl alcohol) the amount of methoxy groups varies between G and S lignins with 14 – 16 % and 16 - 22 %, respectively, which has an influence on the condensation of lignin. Therefore, hardwood lignins are less condensed, which facilitates the degradation (Mai & Zhang, 2023; Windeisen & Wegener, 2012).

2.7.4 Extractives

Wood extractives is a term for substances, which can be solved from the wood matrix by different solvents depending on the solvents polarity. Additionally, this term often refers to substances other than the structural components cellulose, hemicelluloses and lignin, despite the fact that some hemicelluloses or lignin fragments are possibly extractable as well. The fraction of extractives accounts to a minor part of the wood components, with values < 5 % for trees of the temperate zone, dependent to the extraction method. However, there are exceptions as some species like *Quercus spp.* or *Tila spp.* show extract contents >10% (Fengel & Wegener, 2003; Mai & Zhang, 2023).

Extractives can originate from the primary metabolism or the secondary metabolism (heartwood formation) of trees and comprise mostly low molecular weight substances from various substance classes. The proportion of secondary metabolism extractives is notably higher than extractives from the primary metabolism. In general, also inorganic components, referred to as minerals, are classified as extractives, but are presented in a separate chapter (see chapter 2.7.5). Depending on their biosynthesis, the location of extractives within the tree differs. Extractives like amino acids or starch are more abundant in the sapwood region due to their presence in living parenchyma cells and active partition in the trees metabolism (primary extractives). Compared to the sapwood the heartwood region contains a higher extractive proportion. These extractives originate from the secondary metabolism, which converts primary metabolism substances into secondary metabolism extractives like tannins during heartwood formation including the programmed cell death of former living parenchyma cells (Mai & Zhang, 2023).

Especially extractives from the secondary metabolism have important functions, like increasing the resistance of the living tree against fungal or pathogenic attacks. In terms of wood utilization extractives incorporated into the cell wall influence the dimension stability and natural durability of the product. Next to the stem cross section, the extractive content is highest on the trees bottom an decreases to the top and additionally varies between other positions within the tree like roots or branches. Especially the bark shows a notable increased extractive content. Further, the proportion of extractives is dependent on the trees species, the age of the tree and environmental factors, like season or geographical site (Koch, 2006; Mai & Zhang, 2023).

In the following, some important extractive classes are described with focus on hardwoods, as these are most relevant for this thesis. The group of **fats and waxes** are mainly compounds of the primary metabolism and hold a very small proportion in hardwoods. However, waxes are solely present in hardwoods not in softwoods The extractive class of **isoprenoids** comprises terpenes, terpenoids and steroids, which all derive from isoprene units. This group is most prominent for softwoods, but triterpenoids including steroids are also frequent in hardwoods, monoterpenoids and higher terpenes can be present, too. Steroids like the ß-sitosterol are for example common in species like *Quercus, Ulmus*, *Populus* and *Betula*. These substances can for instance also be associated with fatty acids by ester linkages and are known to contribute to the yellowing of pulp after bleaching (Fengel & Wegener, 2003; Koch, 2006; Mai & Zhang, 2023).

The major group of secondary metabolism extractives belongs to the group of **phenolic compounds**, which are also dominant in bark. The commonly used term “polyphenols” comprises substances including one aromatic ring with two hydroxyl groups attached, but is not assigned to one specific group of phenolic compounds. Some of these extractives are a thread to pulping processes. For example some flavonoids or stilbenes inhibit the sulfite pulping process and sterols, fats, fatty acids, waxes or condensed tannins are known to cause pitch problems (Mai & Zhang, 2023). This points out, the importance of sufficient knowledge about these extractives to handle them efficiently on the one side and on the other side it

sharpens the awareness about potential unwanted interaction during biorefinery processes like biological conversion processes.

The **small phenolic compounds like lignin fragment or aromatic acids** comprise a C_6 aromatic ring and a C_1 or C_3 side chain. Common examples are vanillin or syringaldehyde as well as hydroxybenzoic acid and gallic acid or p-hydroxycinnamic aldehyde and p- hydroxycinnamic alcohol. The substance class of **lignans** is based on C_6-C_3 molecule structures like coniferyl alcohol or sinapyl alcohol, which are condensed by ß – ß linkages, at the propanol side chain. The C_6-C_3 molecules can form dimers, but also polylignan structures. Lignans are often linked to sugars forming glycosides. According to the trees lignin type hardwood lignans comprise often syringyl groups. Common examples for hardwood lignans are syringaresinol and lyoniresinol, which are the counterparts to the softwood derived pinoresinol and isolariciresinol. Lignans are frequent in softwood and hardwoods, for example in species from the *Quercus*, *Ulmus* or *Alnus* genera. Like many of the secondary extractives (derived from the secondary metabolism) lignans show some bioactive properties beneficial for the trees defense mechanisms. The **stilbenes** show a C_6-C_2-C_6 structure and are frequent in softwoods, but not very common in hardwoods, except for stilbenes like resveratrol, which is present in *Eucalyptus spp.* and *Monaceae* spp. (Fengel & Wegener, 2003; Koch, 2006; Mai & Zhang, 2023; Sticher et al., 2015).

An important group of phenolic substances is formed by **flavonoids** comprising over 6500 different compounds. Flavonoids are based on C_6-C_3-C_6 structures (diphenyl propane units) and can occur as monomers up to oligomers like condensed tannins. There are several subgroups like flavones, flavonols and respective variations like flavanonole and flavan-3-ol also referred to as catechin. Other subgroups are aurones and chalcones as well as flavan-3,4-diols. Flavonoids are mainly present as individual components, without linkages to sugars. A common flavanol in wood is (+)-catechin. This flavanol and its derivatives like (-)-epicatechin or (+)-gallochatechin are very common within plants. Flavonoids are important compounds regarding the wood durability and some are responsible for the color of wood, next to other substance groups or are even used as wood dyes. Examples for wood dyes would be quercetin or fisetin and morin all belonging to the subgroup of flavonols (Mai & Zhang, 2023).

Tannins represent another big group of phenolic compounds with molecular weights around 500 - 3000 g/ml^{-1}**.** Like flavonoids or lignans, tannins are secondary extractives frequent in the heartwood region or bark. Tannins show properties of bioactivity and are thus associated with the resistance of wood against decay. Further, tannins are linked to the structural components of wood (cellulose, hemicelluloses, lignin), which is an interesting characteristic, valuable for different applications. Tannins can be subdivided into two main groups the condensed tannins and the hydrolyzed tannins. Precursors of **condensed tannins** are flavan-3-ol (catechin) and flavan-3,4-diol. A condensed tannin comprises three to eight flavonoid units. There are different subgroups classifying the condensed tannins by their repeating units, which are connected by C_4 – C_6 or C_4 – C_8 linkages. Polymerized condensed tannins form helical chains and thus the three dimensional structure influences the reaction properties (Hagerman, 1989; Mai & Zhang, 2023). **Phlobaphenes** belong to the group of tannins, but do not show typical characteristics like bioactivity. The **hydrolyzed tannins** occur less frequently in plants than condensed tannins and within the wood species are just found in hardwood species like *Quercus spp.*, *Castanea spp., Juglans spp.* or *Eucalyptus spp*.. Further, hydrolyzed tannins are present in hardwood and softwood barks as well as leaves or grapes (Fengel & Wegener, 2003; Mai & Zhang, 2023). For example, for *Quercus robur/petraea* a tannin content of 3 – 13 % in heartwood and just 1 % in sapwood was reported. Additionally, for *Castanea sativa* a tannin content of 7 – 16 % in 60 to 80 year old trees or 8 %for *Eucalyptus diversicolor* was determined (Wagenführ, 1984).

The biosynthesis of hydrolyzed tannins starts with gallic acid, which bonds by ester linkages to glucose forming galloylglucoses. Pentagalloylglucose is then the precursor of the biosynthesis of gallotannins and ellagitannins, two subgroups of the hydrolyzed tannins (Niemetz & Gross, 2005).

For the biosynthesis of **gallotannins** further galloyl groups are added on the pentagalloylglucose by ester reactions between the hydroxyl group of a galloy group and the carboxylic group of an additional gallic acid, forming depsides. Gallotannins were described to show a disc-like shape, with the galloyl ester groups being arranged along the edges of the molecular surface. The biosynthesis of **ellagitannins** the second subgroup of hydrolyzed tannins, also start from pentagalloylglucose, which is transformed to hexahydroxydiphenoylglucose (HHDP), by oxidative reactions. Ellagitannins are therefore esters of hexahydroxydiphenic acid and gallic acid as well as D-glucose, which are additionally linked by C – C bonds, by oxidative coupling reactions. These additional binding type changes the spatial arrangement and the flexibility and mobility of the tannin. Hexahydroxydiphenic acid is a dimer of gallic acid and forms ellagic acid, by lactone reaction, for instance due to hydrolysis of tannins. Ellagic acid is found in species of *Quercus spp.*, *Juglans spp.* and *Castanea spp.* with proportions of 20 – 60 mg/kg (Fengel & Wegener, 2003; Mai & Zhang, 2023; McManus et al., 1985; Niemetz & Gross, 2005; Zhang et al., 2015).

For *Castanea sativa* and *Quercus petraea* four substances were described to be most important, the vescalagin, castalagin and vescalin and castalin, which were present in different proportion within the species. Interestingly, the chemical composition of chestnut heartwood was proofed to be quite similar to oak heartwood, but not identical, as it seemed that the proportion of gallic acid is higher in chestnut and some other compounds were identified (Sanz et al., 2010; Zhang et al., 2015). Regarding the nature of these extracts, Klumpers et al. (1994) also found, that the polymerization degree of ellagtannins increased with aging of the tree. The group of hydrolyzed tannins is very variable and can for example, form flavon-ellagitannin derivatives, if flavonols are included, one example is acutissimin (Fengel & Wegener, 2003; Zhang et al., 2015).

2,6-Dimethoxybenzoquinone belongs to the group of **quinoids** and was detected in hardwoods. Quinoids are often pigments and have biocidal properties. Another example for quinoids is juglone, detected in *Juglans spp.* showing allelochemical properties and can be further used as dye. However, the group of quinoids accounts just to a small proportion of the possible wood extracts. Lastly, there is the group of **alkaloids**, which show pharmacological activities, but are less important in wood, especially in the temperate climate zone (Mai & Zhang, 2023)

2.7.5 Mineral components

The inorganic fraction in wood is a minor proportion with 0.2 - 0.5 % as typical range in wood of the temperate zone. The proportions of minerals vary within the tree, between tree species and can be influenced by environmental factors, especially through the available soil type (Fengel & Wegener, 2003; Kenney et al., 2013). The mineral content in oak *(Q. robur/petrea)* was determined with 0.3 - 0.6 % and for ash *(Fraxinus excelsior) with* 0.4 - 0.6 %. Further, for beech *(Fagus sylvatica)* the mineral content is detected with 0.3 - 1.2 % and for chestnut *(Castanea sativa Mill.) with* 0.3 - 0.4 % (Wagenführ & Scheiber, 1974). These values are much lower compared to the mineral content of agricultural residues like wheat straw or switchgrass with 8.2 % and 7.7 % (Zając et al., 2018). High mineral contents leads to problems like ash fouling or slag formation during thermochemical conversion. Therefore, woody debarked material is preferred over agricultural residues for thermochemical processes. Ash fouling and slag formation during combustion is based on the presence of alkali metals like sodium or

potassium, especially the latter one is a very abundant mineral in plants (Brown, 2019; Fengel & Wegener, 2003; Kenney et al., 2013).

Physiological minerals are taken up by the tree during its growth and have important functions within the trees metabolism. Next to the physiological minerals there is an **externally introduced mineral fraction**, which is also important as this fraction is assigned to impurities (aluminum or silicates from soils). These are attached to the wood during harvesting, transporting or storage. A tree needs the **macronutrients** calcium (Ca), potassium (K), magnesium (Mg) as well as phosphor (P) and sulfur (S) for its metabolism. Additionally, there is the fraction of **micronutrients**, which are necessary, but just in a small proportion. These are manganese (Mn), iron (Fe), molybdenum (Mo), chlorine (Cl), copper (Cu), sodium (Na), boron (B), zinc (Zn) as well as silica (Si), selenium (Se) and cobalt (Co). As an example the amount in plants (based on dry sprout mass) of the macronutrient potassium is 250 µmol/g and of calcium 125 µmol/g. In contrast, the amount of Mo as micronutrient in plants is 0.001 µmol/g and 0.1 µmol/g for Cu (Brown, 2019; Hörhammer et al., 2018; Kenney et al., 2013; Mai & Zhang, 2023; Matyssek et al., 2010).

Trees take up potassium as K^+ ion from the soils and within the plant, K is involved in the control of the turgor regulation of cells and signal transmitting. Calcium is absorbed as Ca^{2+} and takes over various tasks in the cell, by forming inter- and intramolecular bonds with the cell wall or membranes, which are stable but reversible. Like potassium, Ca is also involved in signal transmission and regulates different mechanisms. Further, the formation of calcium oxalate crystals or the formation of Ca^{2+} protein complexes are typical roles of calcium in the wood cell. Magnesium is an essential element for the trees metabolism as it is part in various enzyme reactions by forming ionic bonds. Next to the already named minerals also phosphor, iron, zinc, manganese or copper are essential elements for the plants metabolism, especially through their interaction with proteins (Matyssek et al., 2010).

2.8 Anatomical features of bark cells and tissue

The bark has various important **protective and metabolism related functions** within the tree. First, the bark protects the wood - body and especially the vascular cambium. The latter one is the meristematic tissue, which forms new wood and bark cells and is located at the interim of wood and bark. The bark works as barrier against fungal, bacterial or insect attacks and compensates the stresses by UV rays, heat and frost. Additionally, the phloem as living tissue of the bark transports and distributes nutrients. The parts of the bark tissue undergoing programmed cell death are also used to dump redundant substances from the trees metabolism by forming for example tannins, oils or mucilages. Another form of waste products stored within bark cells are calcium oxalate or silica based salts, which are typical for beech or lime species. This “waste - product” storage results in an increased content of secondary extractives in bark (IAWA, 2016; Vaucher, 2003).

The final **proportion of bark** varies between the tree parts and is higher for branches or roots than it is for the stem and is dependent to environmental conditions. For the tree stem, guide values were described with a bark to wood ratio of 10 % bark and 90 % wood for an adult tree. The low bark proportion compared to wood is reasoned in the lower amounts of cells formed by the cambium towards the outside and the volume loss due to cell collapse after obliteration, which is explained later (Fengel & Wegener, 2003).

During the first stage of **bark development** the tree shoot cross section is structured as followed: The pith forms the center of the tree, which is surrounded by the vascular cambium as the first meristematic tissue. The vascular cambium forms the xylem to the inside, which is mainly responsible for the water transport. The **phloem** is parallel formed to the outside, mainly responsible for the transport of assimilates. During the first development period, the tissue

outside the phloem is called primary bark or cortex, which is then enclosed by the epidermis. Next to the height growth, the secondary thickness growth is crucial for the trees development. For the thickness growth the vascular cambium generates further cells to the inside and to the outside, which leads to a steady replacement of the fresh xylem and phloem cells and a differentiation of the older cells towards the common wood and bark body. In the case of bark the secondary thickness growth tears the initial epidermis apart, but within deeper primary bark cell layers a new tissue type the **initial periderm** is generated (Fengel & Wegener, 2003; Wagenführ, 1984).

The initial periderm includes a second meristematic tissue the **phellogen**, which forms cork cells (also referred to as **phellem**) to the outside and the **phelloderm** to the inside. The latter one are living cells, which contribute to the phloem, but the cells within the phellem are layered by suberin and cellulose and become non-conductible, resulting in dieback of these and neighboring cells to form dead cell complexes or layers, which are directed to the outside. Dependent on the genetics of the different tree species, some species keep their initial periderm during aging. In this case the periderm forms a thin continuous layer topped by some layers of phellem or cork cells to the outside, resulting in a general thin bark structure, which is for example the case for beech *(Fagus sylvatica)*. This results in a lower resistance against environmental threats. In contrast, for many other species like *Oak spp.*, *Castanea spp.*, *Acer spp.* or *Fraxinus spp.* the initial periderm is teared apart as the tensile force due to the secondary thickness growth cannot be compensated. This results in a continuous formation of new **secondary periderm** layers, leading to the **ring periderm** and **scaly periderm** types. The scaly periderm type is further divided into a flaky and a netlike bark type. The bark proportion containing these secondary periderm layers is called the **outer bark** or **rhytidome** (Fengel & Wegener, 2003; Godet, 2011). Whereas the phloem formed by the vascular cambium towards the outside represents the **inner bark** region, which is bordered by the first secondary periderm layer.

The inner bark phloem contains **parenchymatous cells**, which are living cells and can be grouped into **sieve tubes** (hardwoods), which are connected by sieve plates. Additionally there are **normal parenchyma cells** for the transport of assimilates in longitudinal direction. Phloem rays are responsible for radial transportation. Next, there are **sclerenchymatous cells**. These could be **bast fibers** or **sclereids**. The latter one show thick and lignified cell walls. The bast fibers show a strong lignification of the middle lamella and a thick cell wall, but are elongated in tangential direction with over lapping ends. These bast fibers can appear single, in bundles or tangential rays within the bark. The dimensions of inner bark region can vary between 2 – 15 mm, but the actively assimilating proportion of the phloem is roughly 0.2 – 2.7 mm thick and is located directly next to the cambium. For example, beech or fir show higher inner bark proportion than pine or larch (Fengel & Wegener, 2003; Wagenführ, 1984).

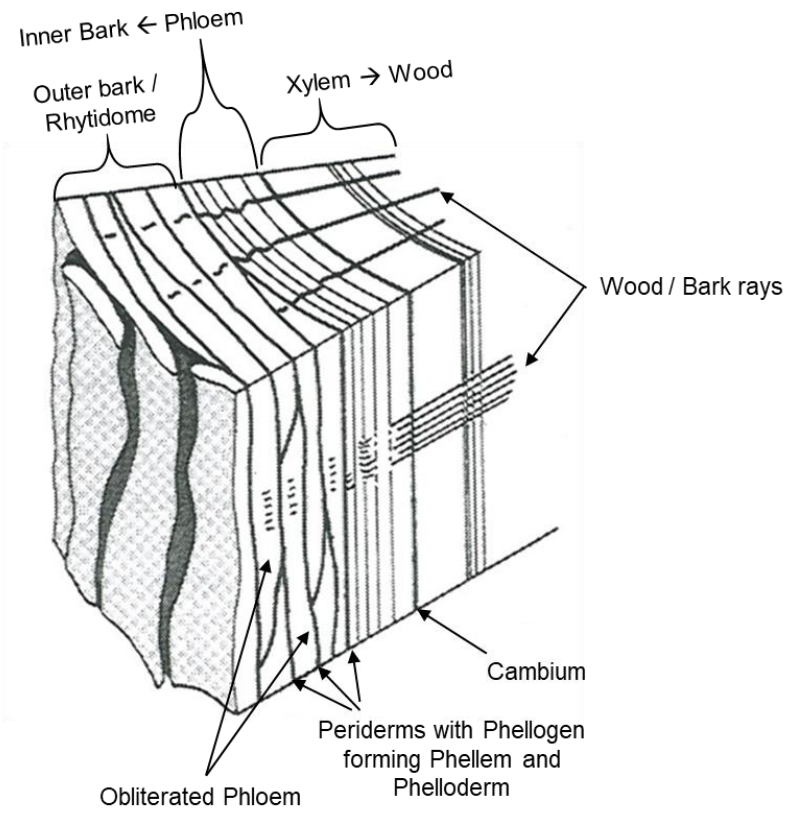


Figure 2: Structure of a scaly bark in adult development stage. Figure is modified based on Figure 9 - 1 in Fengel and Wegener (2003)

Physiological and physical aspects influence the development of bark during **tree aging**. Physiological aspects include mainly the differentiation of cells from living, conducting tissues towards an inactive tissue. With this differentiation called **obliteration** the sieve tubes in the phloem of deciduous trees collapse and the sieve plates are plugged by callose a polyose out of ß - (1→3) - linked glucose units. Further, vertical parenchyma cells became enlarged and metabolic waste product are stored in idioblasts, which are specialized parenchyma cells. The IAWA committee classified **idioblastic cells** as secretory cells, which are specialized parenchyma cells, containing substances like tannins or oils. However, these cells are to be separated from epithelia cells forming resin or gum ducts, the first one very typical for softwoods (IAWA, 2016). The initial tasks of these idoblastic cells are taken over by newly formed living phellogen cells. The physical aspects are related to the appearance of inner pressure and tension forces due to the secondary thickening growth, which leads in some cases to an extension of cells, the formation of new secondary periderm layers or in case of pressure to cell wall thickening (Fengel & Wegener, 2003; Vaucher, 2003; Wagenführ, 1984).

The appearance of the bark during tree aging is dependent on the **bark type**. For beech, which keeps the initial periderm the bark has a thin and smooth structure from shoot to the adult tree. For species like ash, oak and chestnut, which form layers of secondary periderm the bark is first smooth and develops its rough netlike bark structure continuously during tree aging. The time for the development from a smooth to a rough bark structure further varies between the species, for example for ash it can take up to 40 years, until the bark structure changes. For oak, it can take 25 – 36 years to form the “adult” bark structure, for cork oak it even takes up to 50 years, but for pine, it just takes 8 – 10 years. (Godet, 2011; Wagenführ, 1984).

The bark structure contains some information about the **cellular characteristics** as flaky bark types seem to contain less fibers compared to netlike bark types. Typical species with flaky

bark are spruce *(Picea abies)* or sycamore *(Acer pseudoplatanus)*. For species with a netlike structure oak (Quercus robur/petraea), ash *(Fraxinus excelsior),* Norway maple *(Acer platanoides)* or sweet chestnut *(Castanea sativa)* are good examples. Trees which keep their initial periderm like beech *(Fagus sylvatica)* tend to contain no or very less fibers (Moeller, 1882; Wagenführ, 1984). Harder et al. (1978) for example analyzed the bark of 42 different hard - and softwood species. White and green ash showed a high bast fiber content of 15 – 18 %, but a low sclereid content of 1 – 4 %. The fiber content of different oak species varied between 2 – 8 %, but additionally showed a higher sclereid content 8 – 18 %. The values for the American beech show completely contrary picture for the total fiber content which was below 1 %, but had a high sclereid content of 24 %. In contrast the fiber content of softwood species was between 0 – 1 %, but showed a high sclereid content up to 26 %. Holdheide (1970) (cited in Wagenführ (1984)) described the outer bark of *Quercus robur* and *Fraxinus excelsior* as scaly-fibrous bark type and the bark of *Castanea sativa* as scaly bark. Interestingly, the presence of fibers is just described for oak and chestnut, but not for ash. Also for beech *(Fagus sylvatica)* bast fibers are not detected. In contrast, sclerides were detected in all four species. Additionally, the storage of calcium oxalate crystals was described, which show single crystal structures and for *Castanea sativa* and *Quercus robur* also geode crystal structure. For *Fraxinus excelsior,* the crystals were described as sand, which means very small granular crystals. These crystals are present in parenchymatous cells like phelloderm or ray cells and are likely present in the non-conducting phloem tissue (Holdheide, 1970; IAWA, 2016; Wagenführ, 1984).

2.9 Chemical composition of bark

Like in wood, bark contains the main structural components cellulose, lignin and hemicelluloses as well as some pectins (Feng et al., 2013). The chemical structure of polyoses and lignin in bark are similar to wood, but the proportions and composition vary. Bark contains a lower **polysaccharide** content than wood (Fengel & Wegener, 2003). The carbohydrate structure of the bark of some European tree species was determined and a cellulose content of just ~ 26 % and ~ 25 % for beech *(Fagus sylvatica)* and oak *(Quercus robur)* was reported. Based on the monomeric sugar values from this study an O-acetyl-4-O-methylglucuronoxylan content of ~ 25 % and ~ 20 % for beech and oak was calculated. In contrast, the analyzed pine and spruce species showed O-acetyl-galactoglucomannan or arabino-4-O-methylglucuronoxylan, which are not present in hardwood species (Dietrichs et al., 1978).

In case of the **lignin** proportion and variations in bark, a study of an endemic oak species *(Quercus vulcanica Boiss)* for instance showed that the lignin proportion in bark was higher than in the sap- or heartwood of the tree. Additionally the lignin in bark of deciduous trees *(Fagus crenata, Magnolia obovate, Quercus crispula)* showed a lower S/G lignin ratio compared to the respective wood. Further, Dietrichs et al. (1978) determined a methoxy group content of 9.8 % and 13.2 % in beech and oak bark lignin, which differs from the proportions of functional groups in wood lignin.

Bark contains a higher **extract content** than wood, which varies between tree species and the position within the tree. Additionally, similar to wood the extract content is influenced by aging or environmental conditions. Most secondary extractives known from wood are also present in bark, but in different proportions and variations (Fengel & Wegener, 2003).

For example, the extraction of *Quercus vulcanica* with organic solvents or hot water resulted in higher extract proportions for bark than for the wood. The hot water extract content for bark was reported with ~15 % compared to ~9 % for the heartwood. For analyses of *Cedrus libani* the hot water extract content was highest for the proportion of the outer bark > inner bark > heartwood > sapwood, which is in line with the results for the oak species (Usia & Kara, 1997).

A hydrophobic extractive mainly present in bark is **suberin**, which comprises mainly long-chained aliphatic acids and small proportions of glycerol and phenolic acids (hydrolysis products). The presence of suberin in cells mainly targets to prevent water loss and mechanical protection (Fengel & Wegener, 2003; Mai & Zhang, 2023).

Phenolic compounds are one of the most important groups of extractives in hardwood bark. Similar to wood, the subgroup of **tannins** plays a major role in bark, but the proportion of condensed to hydrolyzed tannins varies between wood and bark. A study of Scalbert et al. (1989) for example compared different determination methods for polyphenols (aqueous extract) and reports a total phenol content of 62.6 mg/g for wood of *Quercus robur L.*. For the bark of the same species, a total phenolic content of just 25.2 % was reported. However, the proportion of condensed tannins was notable higher in bark than the proportion of ellagitannins, contrary to the dominating content of ellagitannins detected in the wood (Scalbert et al., 1989).

Like for the extractives also the **mineral content** is much higher in bark than in the wood proportion of the tree. As a guideline, the mineral content in bark is 10 times higher than for wood with Ca as main element, followed by K and Mg. Calcium oxalate crystals are the main form of Ca in bark. For wood also Ca (~ 50 %) followed by K and Mg are the main elements, but in bark, the Ca content is between 82 - 95 %. For American ash and oak values of 12.1 % and 11.1 % are found for inner bark and 0.9 % for the sapwood of both species (Fengel & Wegener, 2003). For the pure bark of *Fagus sylvatica* a mineral content of 4.0 % and for *Quercus robur* of 2.3 % is detected (Dietrichs et al., 1978). For *Quercus vulcanica Boiss* a mineral content of 0.72 % and 0.44 % for the sapwood and heartwood proportion stands against an mineral content of 13.5 % in the bark of the respective tree species (Balaban & Uçar, 2001). The same trend is visible in the study of Usia and Kara (1997), where *Cedrus libani A. Rich* was analyzed, but here even a difference between the outer and inner bark region could be detected with ~45 % and ~34 % mineral content.

C. The disintegration processes of steam explosion (SE) and enzymatic hydrolysis (EH) and influencing factors

2.10 The steam explosion process (SE)

The steam explosion process (SE) is currently one of the most used pretreatment processes to prepare lignocellulosic biomass for biological conversion like enzymatic hydrolysis and fermentation (Berg & Guzmán, 2023). The positive effect of this pretreatment was shown in various studies. For example, the conversion rate (cellulose to glucose) of poplar was increased from 15 % to 90 % after 24 h of enzymatic hydrolysis if the material was first pretreated. Other research similarly found a 10 times increased yield with prior pretreatment (Grous et al., 1986; Schultz et al., 1984). Therefore, the SE process belongs to the first refining step in a biorefinery concept.

For the discontinuous **steam explosion process**, the starting material is filled in a reactor and treated with water vapor, a defined pressure and temperature for a certain time. Generally, the process conditions range between temperatures of 160 - 260 °C, a pressure 0.7 - 4.8 MPa and holding times of 30 s to 20 min (Shafiei et al., 2015). This enables the water steam to penetrate the wood capillary structure. There it first condenses and then heats up the material until it is in equilibrium with the temperature and pressure conditions in the reactor. Through the sudden pressure release to atmospheric conditions at the end of the holding time, the water within the material is explosively decompressed. This leads to a **defibration** of the original wood structure, reduces the **particle size** and increases the **accessibility** for enzymes, supported by the **removal of hemicelluloses** (Kumar et al., 2009; Seidel et al., 2017; Shafiei et al., 2015). This is partly due to the effect of **autohydrolysis**, which is initiated by the cleavage of acetyl groups from the hemicelluloses, which form acetic acid. Glycosidic linkages or ether bonds in hemicelluloses and lignin are then cleaved by the acetic acid (Berg & Guzmán, 2023). Probably, the effect of lignin softening plays a role in the defibration process, too (Blechschmidt, 2010). Donaldson et al. (1988) for instance detected a low removal of lignin, but lignin melting effects during steam explosion of *Pinus radiata*.

After the steam explosion, three main process streams are available: First, the **vapor fraction**, which contains the volatile substances, like furfural as sugar degradation products. Second, a **liquid stream (aqueous)**, containing solubilized hemicelluloses and lignin as well as their degradation products. This stream can contain 40 - 90 % of the hemicelluloses. However, a further fermentation of this stream for ethanol production has not yet reached application in industrial scale. This is reasoned in a lack of suiting microorganisms, which are able to convert a mixture of pentoses and hexoses within a profitable process setup. Lastly, there is a **solid process stream** mainly containing cellulose and residual lignin. One disadvantages of this pretreatment is the formation of degradation products from sugars or lignin, which often have inhibitory properties and can hamper the following enzymatic hydrolysis (Berg & Guzmán, 2023; Kumar et al., 2009; Seidel et al., 2017; Shafiei et al., 2015).

The steam explosion process for itself is not effective enough and thus is likely combined with agents like acid or alkali. For this thesis, the **steam explosion** under **acidic conditions** is most interesting. There are two options, first the material is mixed with 0 - 5 % vaporous SO_2 or second the material is pretreated with liquid sulfuric acid. The severity of the pretreatment can be increased through the presence of the acid, resulting in a better glucose yield, but higher inhibitor production. However, if the formation of sugar degradation products should be kept small the intensity of process conditions can be reduced, showing no improvement in glucose yield, but lower resource input and less degradation products (Shafiei et al., 2015). The pretreatment with liquid sulfuric acid seems suitable for woody biomass, especially for hardwoods, whereas a SO_2 catalyzed process seems to be better suited for softwoods. Typical

temperature ranges are 200 – 220 °C for softwoods and 190 – 210 °C for hardwoods (Shafiei et al., 2015). Kumar et al. (2009) gives as explanation that hardwoods and agricultural residues contain more ether-bonds and less guaiacyl groups within their lignin structures compared to softwoods, which results in less condensed lignin structures (Kumar et al., 2009; Mai & Zhang, 2023).

The steam explosion process was first implemented 1925 by Mason for the processing of a suitable starting material for the fiber board production (Schultz et al., 1983). A low energy need as well as limited use of chemicals and low investment costs make the process economically and environmentally advantageous. Compared to mechanical wood pulping the steam explosion process for example needs 70 % less energy for the particle size reduction. The option to use high biomass loadings and initial bigger particle sizes due to the size reduction during the process is beneficial, too. Therefore, the steam explosion process today is widely used in commercial scale biomass pretreatment (Brethauer & Studer, 2015; Daraei Garmakhany & Sheykhnazari, 2017; Jacquet et al., 2015; Kumar et al., 2009; Seidel et al., 2017). Pretreatments like ammonia fiber expansion (AFEX), which use alkaline solutions to penetrate the biomass and an explosion reaction like the SE process or the liquid hot water process (LHW), which mainly uses hot water under pressure to initiate autohydrolysis are also under investigation. However, these pretreatments are not well suited for woody biomass due to the high lignin content of wood, which is disadvantageous for the AFEX process or the need of low solid loadings for the LHW process, which reduces economical profitability (Shafiei et al., 2015).

Since implementation the process was tested for various kinds of lignocellulosic biomass as the efficiency of the SE process is highly dependent on the type of raw material, the chosen target product and thus the applied process conditions (Shafiei et al., 2015). Different researchers investigated materials like agricultural residues especially bagasse and straw, but also different forest residues like olive tree pruning or hardwood chip mixtures. Additionally, different European, Japanese, Mediterranean or American wood species were used in different research approaches. Probably most interesting for the present study approach are the researches on spruce, poplar, German beech or some oak species (Balan et al., 2020; Cotana et al., 2015; Grous et al., 1986; Pielhop et al., 2016; Schultz et al., 1989; Schultz et al., 1984; Seidel et al., 2017)

2.11 The process of enzymatic hydrolysis (EH)

The prior described steam explosion prepares the lignocellulosic material for the main process step of biological conversion, which is the **saccharification**. The saccharification includes the conversion of polymeric sugars, mainly the cellulose, into the individual sugar monomers. These sugar monomers are the starting material for subsequent biological processes like fermentation. A common method for saccharification is **enzymatic hydrolysis (EH)** (Berg & Guzmán, 2023; Raghavendra et al., 2016). The saccharification process thus belongs to the first refining step, like the SE process, as it is the main step to produce platform components for further syntheses (FNR, 2012).

From the perspective of practical application the principal progress of an EH can be described as followed: A process stream, which is rich in polymeric sugars, like cellulose (for example the solid process stream after SE) is filled in a reactor (in laboratory this equals to a closed container). In the reactor, the solid material reacts in an aqueous solution with the enzymes under controlled temperature and pH conditions and continuous mixing.

EH is a heterogeneous reaction with multiple steps to break down the inter and intra connected cellulose structure towards monomeric glucose molecules. The reaction takes place at the interface of the solid material and the liquid reaction medium containing the solved enzymes

(Berg & Guzmán, 2023). Therefore, there are three general reaction steps for an EH: first is the adsorption of the enzymes from the liquid medium to the substrate, second is the actual conversion reaction “cleavage” and third is the desorption of the enzyme from the substrate back into the reaction medium to move to the next reaction site (Sun & Cheng, 2002).

Mostly enzymes are very specific regarding preferred reaction conditions and the reaction mechanism including substrate and the type of linkage, which will be cleaved by the specific enzyme. Therefore, a balanced mixture out of different enzyme types is necessary, which work synergistically to cleave cellulose to monomeric glucose units. Additionally, in industrial applications mostly enzyme blends are used, which not only contain enzymes suitable for cellulose, but additional enzymes for example suitable to cleave hemicelluloses (Raghavendra et al., 2016).

There are three enzyme groups necessary to effectively cleave cellulose to glucose monomers, the group of exoglucanases, which include glycanohydrolases and cellobiohydrolases (CBHs), the group of endoglucanases (EG) as well as the group of ß- glucosidases (BGLs) (Annamalai et al., 2016b; Kumar et al., 2008; Raghavendra et al., 2016)

Within the group of exoglucanases the **cellobiohydrolases (CBHs)** are most important. The CBHs target the crystalline cellulose regions. The enzymes attach to free ends of the cellulose chain by their carbohydrate binding modules (CBMs), which secure a tight fit between enzyme and substrate. The CBM is wedge-shaped and has a hydrophilic and a hydrophobic area, suitable to adsorb on the cellulose surface. The actual reaction happens in the catalytic domain of the enzyme, which has a tunnel shape to slide the cellulose chain through, towards the active site, where the reaction takes place processivley. A linker connects the CBM and the catalytic domain. The main cleavage product of CBHs is cellobiose. CBHs can be subdivided into two groups CBH I and CBH II, which differ in their reaction mode. Whereas CBH I catalyzes the cleavage of glycosidic bonds from the reducing side, CBH II does the same but from the non-reducing end of the cellulose chain. Interestingly, the activity of CBHs shows product inhibition, due to a reduced activity, if the cellobiose concentration becomes too high (Annamalai et al., 2016b; Eckard, 2015; Raghavendra et al., 2016).

Endoglucanases (EG) randomly catalyze the cleavage of ß-(1→4)-glycosidic linkages of cellulose chains, mainly within amorphous regions of cellulose. Through the random cleavage activity, new free cellulose chain ends are available, which work as starting point for CBHs. There are various different kinds of EG, some containing carbohydrate binding module (CBMs) others not. The actual cleavage happens in the catalytic core or active site of the EG enzyme, which has a groove or cleft like structure, suitable to attach to a cellulose chain (Annamalai et al., 2016a).

The third enzyme group are the **ß - glucosidases (BGL)**, with a more barrel like catalytic core. The BGLs cleave the cellobiose to monomeric glucose units, but at the same time BGLs show product inhibition towards glucose (Ouyang & Xu, 2016).

The presented cellulase groups work synergistically as the CBHs are necessary to cleave the crystalline cellulose proportion, but depend on the availability of new free chain ends, which are provided by the EGs. Further, the BGL finally cleave the cellobiose to glucose monomers and prohibit inhibition of the CBHs due to cellobiose (Annamalai et al., 2016b). Figure 3 visualizes the reaction mechanism of the different cellulases on cellulose chains.

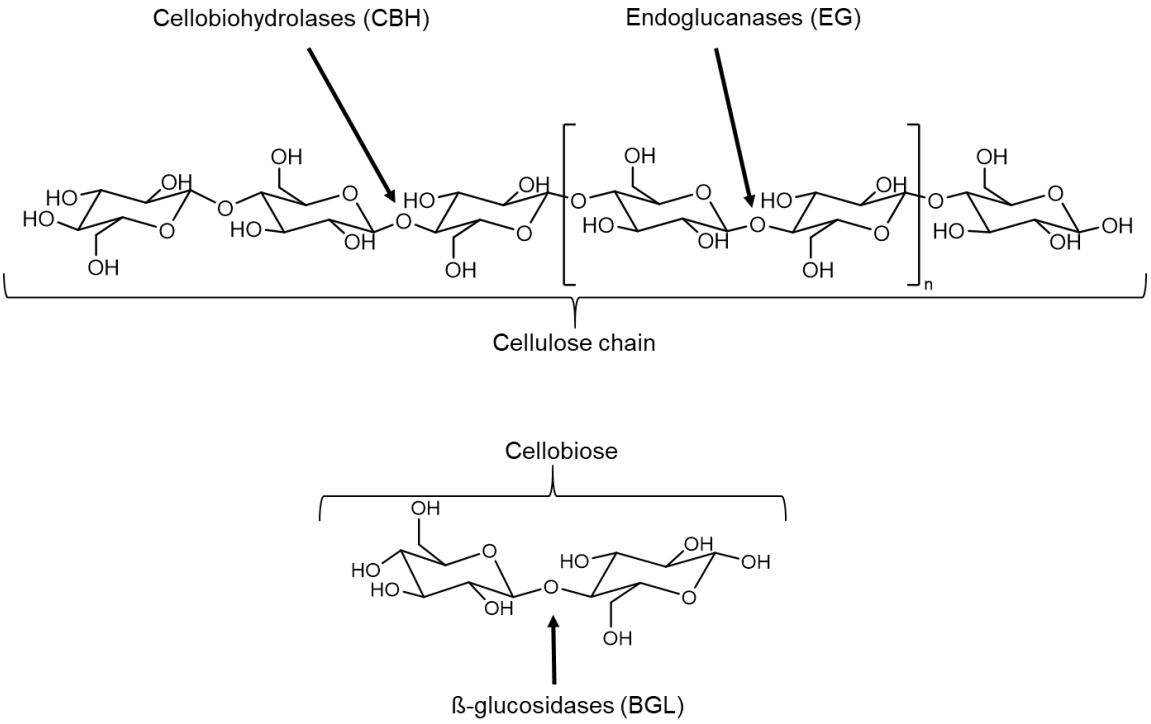


Figure 3: Reaction mechanism within a cellulase mixture for the catalyzed conversion of cellulose to glucose; adapted from (Kumar et al., 2008)

For the conversion of different hemicellulose sugars, different kinds of enzymes are in charge, depending on the hemicellulose structure, for example their backbone molecule with mainly xylose for hardwoods and mannose for softwoods. These enzymes are able to cleave the hemicellulose backbone. Prominent **hemicellulases** are endo-1,4-ß-xylanases, ß-xylosidase, ß-mannanase, ß-mannosidase, α-glucuronidase, α-L-arabinofuranosidase and acetylxylan esterase. Next to cellulose and hemicelluloses also the other wood components like pectins and lignin can be targeted by enzymes (Berg & Guzmán, 2023; Kumar et al., 2008). As the enzymatic conversion of these groups is not the main focus of this thesis, the respective enzyme systems are not further described here.

The composition of the cellulolytic complex differs regarding the **microorganism it is originated from**. For instance, the cellulases from *Trichoderma reesei (T. reesei)* contain a high amount of endo- and exoglucanases, but less ß-glucosidases. In contrast, the cellulases from *Aspergillus niger (A. niger)* contain a higher proportion of the latter ones. The effectiveness of the enzymatic hydrolysis is thus depending on the suitability of the enzyme system used for the specific application including factors as substrate characteristics and the synergistically effects between the main enzyme groups to prevent product inhibition. However, a **mixture of different cellolytic enzymes** always works more effective than a single enzyme type (Irwin et al., 1993; Yuan et al., 2021). The origin of the cellolytic enzymes and specific enzyme engineering influence the characteristics of the final enzyme blend used for specific application (Raghavendra et al., 2016). Even a specific substrate sensitivity of cellolytic enzymes in regard to their origin is described in literature (Ko et al., 2015b)

The conversion by enzymatic hydrolysis is a common method in biological conversion processes, but has some **draw backs** and is thus seen as the critical step in the process chain. The enzymatic conversion of biomass is in general challenging due to the high complexity of the material. Different pretreatment options are one way to increase the convertibility of the materials. However, there are more limiting factors, like the limited **activity range** of enzymes, in regard to optimum temperature (45 – 55 °C) and pH (4 – 5), but also factors like enzyme load and solid load play an important role, next to other inhibiting factors. Especially, the

enzyme load is a critical factor, as on the one side a high enzyme load leads to insufficient costs and potentially low yields due to over crowed free sites at the cellulose, resulting in a slow conversion. On the other side, a too low enzyme load does not represent the maximum and thus economical feasible yield and increases the necessary residence time. Another critical factor is the **solid load**, as contrary to the expectations higher solid loads do not automatically result in higher yields. If the amount of water in the reaction is too low, the viscosity increases, which impedes the mixing. Additionally, by increasing the solid load, less water is available as reaction medium and to distribute the reaction products, thus product-induced inhibition becomes more likely (Berg & Guzmán, 2023; Eckard, 2015; Talebnia, 2015).

Therefore, the correct management of the process conditions in terms of temperature, pH as well as enzyme and solid load are crucial factors. Next to these factors, there are some more influencing factors, which are not directly linked with the process conditions during enzymatic hydrolysis. Some of these will be discussed in the following chapters, considering the influence of the SE process.

2.12 Factors influencing the EH in relation to the SE process

2.12.1 Particle size

The **particle size of the raw material** is one factor, which has a direct influence on the SE process, as the pore system of wood and the particle size seem to influence the processes during SE (Brownell et al., 1986; DeMartini et al., 2015; Wang et al., 2015).

Two possible theories for the heating process were detected. First, the fast entering and diffusion of the steam through the vessels, until the water condenses, which is possible if the vessels are not blocked by tyloses. Second, if the vessels are already filled with water or other impregnation liquids a slow conduction happens (Brownell et al., 1986). DeMartini et al. (2015) similarly emphasized that the natural pore structure is the major transport system for the hydration of wood chips. Therefore, even processes like chipping or the wood storage conditions, which potentially influence the wood conduit system, may subsequently affect the SE process and the following EH. Further, a dependence of pretreatment efficiency on the chip thickness was found (DeMartini et al., 2015). During pretreatment, the water vapor potentially needs longer to penetrate big particles sufficiently, leading to heterogeneous heat distribution. The heat distribution was further dependent on the initial water content of the material and the heating time increased with the moisture content (Brownell et al., 1986).

The intensity of biomass deconstruction in terms of the defibration due to SE is additionally related to the biomass type as softwood pulp showed a higher sensitivity than hardwood pulp and cotton linter showed an even lower sensitivity. (Wang et al., 2015). Ballesteros et al. (2000) tested different chip sizes of *Pinus pinaster* for their enzymatic digestibility after steam explosion and found the best cellulose and hemicellulose recoveries and good digestibility for the biggest tested chip sizes of 8 – 12 mm. Interestingly, Negro et al. (2003) found no influence of the particle size (2 – 5 mm and 12 – 15 mm) with material form *Populus nigra* after SE in relation to enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF). Solely the glucose recovery was significantly dependent to the particle size in relation to temperature. Additionally, smaller particle sizes showed a slightly higher xylose concentration in the liquid process stream after SE (Negro et al., 2003).

The **particle size of the solid process stream** is a direct consequence of the performance during SE and thus related to the particle size of the raw material. Seidel et al. (2017) investigated the influence of the explosion step at the end of the SE process. Whereas, for the pretreatment of corn stover the positive effect could not be proved continuously, the enzymatic digestibility of beech was successfully increased through the explosion. For example, a

treatment of 10 min at 15 barg steam pressure without explosion reached a conversion rate of ~ 43 %, but with explosion it was increased to ~ 68 %. Further, the absolute pressure was identified as relevant factor, influencing the enzymatic digestibility. A study using spruce as raw material supports this finding (Pielhop et al., 2016). It seems that the effect of particle size reduction and thus an increased accessibility for enzymes, through an increased amount of holes (100 – 10 nm) and rearranged fibers, are the main benefits of the SE process (Pielhop et al., 2016). This was supported by the missing positive effect of the explosion at lower temperatures and pressures, which had no effect on the particle size, too. However, higher intensities effective reduced the particle size and resulted in a higher enzymatic digestibility (Pielhop et al., 2016). The influence of particle size reduction was further proven by experiments with varying enzyme loads and material with and without explosion step of similar size. The digestibility decreased with raised particle size and was the same for particles of the same size independent to the explosion step (Pielhop et al., 2016). In contrast, the study of Brownell et al. (1986) on aspen wood *(Populous tremuloides)* did not found a positive effect of the explosion step, which can be reasoned in the different properties of the tested biomass or discrepancies in the application of the enzymatic hydrolysis.

Regarding the actual particle size, in the study of Pielhop et al. (2016) the rate of the particle size decrease flattened in the region of higher pretreatment intensities and a minimum average particle size of 0.4 mm mesh size was observed. Within a pretreatment at 235 °C, a holding time of 5 min and a pressure difference of 30 bar the cumulative particle-size distribution showed a sharp increase around the mesh size of 0.02 mm, but the strongest cumulative rise was in the region of 0.1 mm and 1.4 mm mesh size (Pielhop et al., 2016). The study of Seidel et al. (2017) presented the cumulative particle distribution of experiments with wet sieving of steam exploded beech, which showed the strongest rise between particle sizes of 0.5 – 1.0 mm for pretreatment conditions of 230 °C for 15 min and varied pressures between 7 to 27 barg (Seidel et al., 2017).

Overall, it seems that one important effect of SE is to reduce the particle size. However, if the process conditions are suited to reach deconstruction of the material, there is still a difference detected between the raw materials, which is possibly related to the conduit or pore system of the respective lignocellulosic material.

2.12.2 Substrate accessibility

The accessibility of the material for the enzymes is described as an important factor for a sufficient EH (Eckard, 2015) and is cross-linked with the prior discussed aspects of particle size. As described prior the steam explosion process reduces the particle size and dissolves the hemicelluloses as well as likely softens the lignin structure or redistributes the lignin. These are processes, which probably increase the accessibility, by reducing the shielding of the cellulose fibers through hemicelluloses and lignin. Additionally, the reaction surface is increased due to smaller particles and increased pore surfaces, because of the removed hemicelluloses and new openings, which were potentially caused by the rapid vaporization during the explosion step (Berg & Guzmán, 2023; Donaldson, 1988; Donaldson et al., 1988; Pielhop et al., 2016; Shafiei et al., 2015). For instance, Donaldson et al. (1988) investigated the ultrastructural changes in *Pinus radiata* during steam explosion. It was found that the cell structure was mainly disrupted into two main fractions. The first fraction comprised the middle lamella, the primary cell wall and the S1 layer of the secondary cell wall. The second fraction solely comprised the secondary wall. Therefore, the fractionation led to a decrease of permeability barriers, as for example the middle lamella has high lignin contents. Further, especially the S1 and S3 layer of holocellulose fibers showed high hemicellulose contents, resulting in small pore sizes and thus a hampered permeability for enzymes during enzymatic hydrolysis (Donaldson et al., 1988).

However, the term accessibility comprises different **structural aspects**. For example Wang et al. (2012) and Luo and Zhu (2011) emphasized that the outer **particle surface** is less important to evaluate the accessibility, but the inner **pore surface** seems to be an important value. Further, there is a potential correlation of the **pore size distribution** with the enzymatic digestibility (DeMartini et al., 2015). Additionally, the **shape of the pores** is influencing the accessibility for enzymes, as for example a slim bottle neck or a missing opening of a pore limit the accessibility (Suurnäkki et al., 1997).

The different structural aspects point out the complexity behind the topic of substrate accessibility. The characteristics of lignocellulosic materials are especially challenging for the evaluation of this value. A common method to evaluate the accessibility in terms of surface area is the **Brunauer-Emmett-Teller (BET)** method, which measures the adsorption of N_2 at the external material surface and within the pores by determining an adsorption isotherm at constant temperature and changing vapor pressure (Beecher et al., 2009; Fagerlund, 1973). The **water retention value (WRV)** determines the pore volume, by the ratio between the weight of wet material after centrifugation and oven dry material, to measure the amount of water retained in the pores (SCAN-C, 2000). The **NMR porosimetry** uses the different diffusion rates of free and cell bound water to determine the pore volume (Beecher et al., 2009). The **Simons staining** method determines the pore surface by measuring the absorbance of different dyes to the substrate and was for example applied by Luo and Zhu (2011). The **solute exclusion** method determines the pore volume by mixing wet material with a solution of a known substance (solute molecule), molecule size and concentration. By evaluating the change in the solute concentration, it can be determined how much water from the pores is inaccessible to the osmotic concentration exchange. The proportion of the water, which is not accessible, depends on the size of the solute molecule and its potential to access the pores of different sizes (Grethlein, 1985; Wang et al., 2012).

About these methodologic approaches and the characteristics of lignocellulosic materials in relation to enzymatic digestibility, some aspects are to consider. For example, for the BET method, the material has to be dried prior to measurement. However, it is well known, that **drying** can lead to **shrinkage**, **pore collapse** or **hornification** effects in wood related structures, which are irreversible (Thybring & Fredriksson, 2023). Therefore, re-soaked, freeze dried or air dried samples likely underestimate the specific pore surface area (Hill & Papadopoulos, 2001; Kang et al., 2018). Methods like solvent exchange drying (SED) or super critical point drying (CPD) were tested as alternatives drying methods for the sample preparation for BET analysis. For SED the water is stepwise removed by washing the sample with different solvents, for example ethanol > acetone > toluene. With this alternative method the pore volume of milled scots pine wood, determined by BET with N_2 absorption, could be increased from 0.0021 cm^3/g if the sample is oven dried to 0.013 cm^3/g using SED with toluene as last solvent. The choice of the last solvent seems to influence to final pore size and is probably dependent on the material type. However, the collapse of microvoids was still detectable after removing the last solvent (Hill & Papadopoulos, 2001; Papadopoulos et al., 2003). The CPD method is a very gentle drying method but still needs some solvent exchange steps prior to the final drying (Hill & Papadopoulos, 2001; Papadopoulos et al., 2003). Additionally, the washing of sample with different solvents like ethanol or acetone potentially leads to an **extraction** of the material (Mai & Zhang, 2023), which could possibly led to a distortion of the real pore volume or change the accessibility due to a reduced shielding of cellulose fibers through extractives. Due to the drying effect during sample preparation the BET method seems to be not suited to evaluate the accessibility in regard to enzymatic digestibility (Beecher et al., 2009).

Another aspect is the **size of the molecules**, used to determine the substrate accessibility. N_2 molecules or water molecules are smaller than a cellulase enzyme (5.1 nm, saturated sphere) (Grethlein, 1985). Additional to the general limitation of the molecule size, the **shape of the**

pores is influencing the practical accessibility, which is also dependent on the molecule size. Therefore, WRV or NMR porosimetry would be good alternatives to circumvent the drying effects, but the determined pore volume is potentially also not representative, because it overestimates the pore volume, which is actually accessible to the enzymes (Suurnäkki et al., 1997).

The solute exclusion method uses wet material and considers the aspects of molecule size and pore shape. Literature shows a positive relation with the cellulose conversion during enzymatic hydrolysis and the results of solute exclusion method (Grethlein, 1985; Wang et al., 2012). The method was used for various kinds of pretreated material originated from Kraft, sulfuric acid, sulfite or steam explosion pretreatments and was also tested for different tree species like spruce, birch, sweetgum poplar and pine (Grethlein, 1985; Grous et al., 1986; Stone & Scallan, 1968b; Suurnäkki et al., 1997; Wong et al., 1988). However, the **adsorption** of the solute molecules to the substrate or the electrostatic charge of the solute molecules and possible **electrostatic repulsion** as well as the **spherical behavior** in water are factors potentially influencing the measurements (Beecher et al., 2009; Hill & Papadopoulos, 2001). In literature, mostly cross-linked dextrans were used, but also polyethylene glycols were tested (Grethlein, 1985; Grous et al., 1986; Ishizawa et al., 2007; Stone & Scallan, 1968b).

Additionally, there were some approaches using a nonhydrolytic fluorescence fusion protein with a CBM unit similar to the size of cellulases to determine the accessibility of the substrate. However, the **adsorption of proteins** on the residual lignin is another aspect common for lignocellulosic materials, which need to be considered (Hong et al., 2007; Wang et al., 2012).

2.12.3 Liquid fraction

Hemicelluloses

During the steam explosion process, a part of the raw material is dissolved. This proportion is included in the liquid process stream. The largest share is taken by **dissolved hemicelluloses**. The amount of recovered hemicelluloses is dependent on the raw material and the chosen process conditions. However, there is a discrepancy between the best conditions for optimized cellulose digestibility and maximized hemicellulose recovery. Higher severities mostly lead to an increased cellulose digestibility, but at the same time to an increased sugar degradation, reducing the hemicellulose recovery (Shafiei et al., 2015).

Ballesteros et al. (2000) for instance, investigated the influence of different particle sizes of *Pinus pinaster* wood chips under varying process conditions in relation to cellulose digestibility and hemicellulose recovery. It was shown that up to a particle size of 8 mm the hemicelluloses were mainly dissolved even at low temperatures of 190 °C, but for larger particles, an increased temperature was necessary to reach suitable hydrolysis. It was further found, that an increased temperature and residence time increased the hemicellulose degradation, but a bigger particle size could decrease that effect.

Negro et al. (2003) investigated *Populus nigra* under different steam explosion conditions and detected the lowest share of hemicelluloses in the solid proportion during SE at 210 °C with a residence time of 8 min. However, the xylose recoveries (main proportion of hardwood hemicelluloses (Mai & Zhang, 2023)) were with 5.7 g per 100 g poplar biomass (2 – 5 mm particle size) the lowest detected values, probably due to sugar degradation. In contrast, the lowest intensities with 190 °C and a residence time of 4 min recovered the highest xylose proportion with 8.6 g per 100 g poplar material (2 – 5 mm particle size), but parallel showed the highest residual hemicellulose proportion in the solid fraction (Negro et al., 2003).

The dissolving rate seems to be dependent on the process conditions in relation to the chip size. However, there is less information about, if these hemicelluloses are available as

monomeric sugars or are present as oligomers. Negro et al. (2003) applied a mild acid hydrolysis on the liquid process stream and reported the individual monomeric sugars. Kim et al. (2011) detected xylo-oligomers and monomeric xylose in the liquid fraction after hot water pretreatment (LWH) of maple. Additionally, several studies detected an inhibitory effect of xylo-oligomers and monomers during enzymatic hydrolysis (Kim et al., 2011; Ximenes et al., 2010).

Sugar and lignin degradation products

The liquid process stream contains some **lignin** and **sugar degradation products**, next to the hemicelluloses (Kim et al., 2011; Shafiei et al., 2015). Some studies evaluated the liquid stream after different pretreatments like steam pretreatment and steam explosion. The controlled addition of the liquid process stream to subsequent biological conversion processes showed a decrease in enzymatic conversion and especially an inhibitory effect on ß – glucosidases. This point out, the importance of a sufficient separation of the liquid and solid process stream and suited detoxification methods for the liquid process stream (Kim et al., 2011; Mes-Hartree & Saddler, 1983; Shafiei et al., 2015).

Negro et al. (2003) investigated different aspects of the conversion of *Populus nigra* with steam explosion and detected an increasing proportion of sugar degradation products with rising process intensity. At the highest intensity (210 °C, 8 min, particle size of 12 – 15 mm) about 2.9 g/100 g of poplar biomass of acetic acid and 0.53 g/100 g of poplar biomass of formic acid as well as 0.94 and 0.22 g/100 g of poplar biomass of 2-Furfural and 5-HMF were detected (Negro et al., 2003). Kim et al. (2011) analyzed the liquid process stream after liquid hot water (LWH) pretreatment of maple and found sugar oligomers and monomers next to 13.1 g/l acetic acid as well as 4.1 g/l furans (HMF, furfural). Additionally, 1.3 g/l of phenolic compounds were detected. During following experiments about the inhibitory effects of the different components, it was shown that oligomer and monomer xylose sugars hampered the enzymatic hydrolysis. However, for acetic acid and the furans within the detected concentration no impeding effect could be detected (Kim et al., 2011). However, especially furfural and HMF are commonly known to hamper enzymatic hydrolysis (Talebnia, 2015). Regarding the phenolic compounds, with a ratio of 0.5 mg phenolics per mg of total protein, about 60 % of the protein was precipitated within one hour, which increased to 75 % after four hours (Kim et al., 2011).

Tejirian and Xu (2011) tested different monomeric phenolic substances for an inhibitory effect during enzymatic hydrolysis. Simple phenolic substances are for example vanillin, coniferyl alcohol or aldehyde, ferulic acid or syringaldehyde, which are typical degradation products of lignin. These substances showed no decrease of enzymatic conversion at a concentration of 1 mM. At a concentration of 10 mM, syringaldehyde as well as 4-hydroxy-3-methoxy-α-methylbenzyl alcohol showed a strong negative effect on enzymatic conversion (Tejirian & Xu, 2011).

Ximenes et al. (2011) investigated the inhibition and deactivation effects of different phenols. The inhibition effect was thus measured by the reduced activity of the enzymes directly after mixing of the phenols with the enzymes and the substrate and the deactivation effect was represented by the loss of activity after 24 h of incubation. The phenols cinnamic acid, ferulic acid, p-coumaric acid, sinapic acid, vanillin, syringaldehyde and 4-hydroxybenzoic acid showed nearly no inhibiting effect, which is in line with the findings of Tejirian and Xu (2011) at least at low concentrations. However, after 24 h of incubation the phenols cinnamic acid, 4-hydroxybenzoic acid as well as vanillin and syringaldehyde showed deactivation effects for CBHs, EGs and ß-glucosidases. For the ß-glucosidases of *T. reesei* for example p-coumaric acid and sinapic acid the deactivation of cellobiase activity was ≥ 40 % with a deactivator concentration of 0.3 mg per mg protein. Interestingly, the deactivation of ß-glucosidase of *A. niger* was < 40% for coumaric acid and even lower for sinapic acid (Ximenes et al., 2011).

Overall, the formation of phenolic compounds and sugar degradation products is dependent on the substrate and the pretreatment type as well as the process conditions. The concentration and type of degradation products strongly influences the inhibitory effect. Additionally, the enzyme type and the origin of the enzymes, for instance produced by *T. reesei* or *A. niger* seemed to have an influence on the sensitivity regarding inhibition (Negro et al., 2003; Ximenes et al., 2010, 2011).

2.12.4 Solid fraction

Cellulose

The solid process stream after steam explosion contains cellulose and lignin as main components (Shafiei et al., 2015). The accessibility of cellulose is the basic requirement for a successful bioconversion and can be described by its macro- and micro-accessibility.

The **macro-accessibility** focus on the cellulose fibers, which can be **shielded** by hemicelluloses and lignin. The elementary fibrils of cellulose are related to the **micro-accessibility** and comprise mainly the parameters of **chain length** (degree of polymerization (DP)) (Bednar & Fengel, 1974)) and **crystallinity** (Shafiei et al., 2015). The chain length is related to the sensitivity of the cellulose chain towards degradation and increases with decreasing chain length. The amorphous regions of cellulose are hydrolyzed faster than crystalline regions due to their less orderly structure and better water and enzyme adsorption. The main object of the crystallinity is the layered structure, which forces the enzyme to degrade it layer by layer. Additionally, crystalline structures lead to less hydrophilic properties, which emphasize the irreversible binding of cellulases and a decreased activity (Shafiei et al., 2015).

However, the evaluation of the influence of cellulose chain length and crystallinity is challenging, as these properties cannot be analyzed individually. To determine the chain length the cellulose has to be isolated and these separation methods already have an influence on the DP, which therefore varies and is dependent to the isolation method. In the case of cellulose crystallinity, correlations of digestibility and crystallinity, reported in literature are overlapped or cross-linked with other factors for instance an increased accessibility or removal of hemicelluloses (Fengel & Wegener, 2003; Shafiei et al., 2015).

Puri (1984) found an increased cellulose crystallinity after SE of eucalyptus and pine, but this is traced back to the decrease of amorphous sites and thus an increase of the crystalline proportion, which is not equal to a real increase of crystallinity. Also Mansfield et al. (1999) described the contrary discussion in literature about the influence of cellulose crystallinity on enzymatic digestion and points out that other factors may play a more important role (Mansfield et al., 1999).

Lignin – reactions during steam explosion

The solid process stream after steam explosion (SE) comprises the main proportions of cellulose and lignin, as lignin is not removed during SE. However, the lignin seems to undergo some changes during the process, which potentially influences the subsequent enzymatic hydrolysis (Donaldson et al., 1988; Shafiei et al., 2015).

Donaldson et al. (1988) investigated the ultrastructural changes in steam exploded *Pinus radiata*. This study detected the dissolving of the hemicelluloses and a very small removal of lignin, but a strong **redistribution of lignin** was detected. The redistributed lignin particles were detected within porous cell wall medium either attached or incorporated and appeared with a more or less uniform size and spherical shape. However, these lignin particles seemed to be not actively bound to the cellulose fibrils at this state. The authors related this effect to

lignin melting during the pretreatment at high temperatures followed by a subsequent coalescence during cooling forming dense lignin particles. Interestingly, the lignin within the middle lamella showed no degradation or redistribution effects (Donaldson et al., 1988). The study of Pielhop et al. (2016) investigated the influence of the explosion step in steam explosion pretreatment of spruce material. Specific lignin allocations on the pretreated material in the shape of lignin droplets could be solely detected, if there was no explosion step. However, these lignin droplets were not present if the explosion step was included in the pretreatment. These lignin droplets potentially can influence the EH (Pielhop et al., 2016).

During steam explosion, the lignin for itself undergoes **depolymerization and recondensation** reactions, which are initiated by autohydrolysis of the acetyl groups aligned to the polyoses and resulting in a more acidic pH. During the pretreatment, the cleavage of ß – O – 4' linkages is most present, leading to depolymerization (Berg & Guzmán, 2023; Li et al., 2009). This reaction is started at the benzylic position by removing the hydroxyl groups due to a nucleophile reaction partner and result in the formation of a carbocation/quinone methide as an intermediate structure. The further removal of a proton leads to the formation of enol ethers, which are hydrolyzed to oxonium ions. These labile structures react with H_2O resulting in the formation of Hibbert ketones (Li et al., 2009; Miles-Barrett et al., 2016). Repolymerization is thus initiated through the electron rich C – 2 and C – 6 positions in guaiacyl- and syringyl-rings and competes with the prior described degradation reaction. The repolymerization leads to higher condensed lignin structures with higher proportion of penta-substituted aromatic rings, resulting in a lower degradability. Applying the process under acidic conditions, enhances that effect further as the more acidic conditions lead to a higher proportion of degradation and repolymerization reactions (Li et al., 2009). Shimizu et al. (1998) investigated the total utilization of wood applying SE pretreatments and it was found that the process mainly affected the S2 layer of the secondary cell wall. Additionally, it was observed that the Klason-lignin yield is correlated with the lignin type. Therefore, a higher proportion of G-lignin increases the Klason-lignin yield and a higher S- lignin yield reduces the yield. It was further indicated, that S-lignin degradation is enhanced during the steaming and refining. Additionally, Jakobsons et al. (1995) detected an increased removal of syringyl units during pretreatment if the process conditions were intensified.

The alkaline extractable lignin of birch *(Betula verrucosa)* and aspen *(Populus tremula)* after (acidic catalyzed) steam explosion were characterized by Li et al. (2009). It was determined that the molecular size (Mp) with 3091 Da and 2871 Da of birch and aspen after SO_2 catalyzed steam explosion was lower than for wood. Additionally, it was observed that the acid catalyzed process showed the strongest degradation effect, but the highest polydispersity values around 25 and 38 for birch and aspen lignin (Li et al., 2009). Based on the determined proportions of G- and S-lignin units, S/G ratios of 5.1 for birch and 2.9 for aspen were determined. Further evidence for the change in lignin structure during steam explosion is shown by the decreased intensity of the FTIR signal at 1725 cm^{-1} for ester groups and the increase at 1700 cm^{-1} for carbonyl groups. Further, an absorption peak at 1695 cm^{-1} indicates the formation of keto groups and increasing signals at 870 cm^{-1} and 890 cm^{-1} relate to the penta-substitution due to lignin condensation. It was indicated that the SO_2 catalyzed process showed the highest proportion of condensation. Steam explosion further seemed to reduce the aromatic methoxy groups related to a change in the S/G lignin ratio and showed a decrease in aliphatic –OH groups. The proportion of phenolic hydroxyl groups as well as of carboxyl groups is increasing, the first one most pronounced for the acidic-catalyzed steam explosion (Li et al., 2009).

In the work of Lomax et al. (1994) changes in the bark of *Pinus radiata* during steam explosion were investigated and NMR measurements detected a decrease in the ratio of lignin material to tannin material. As a result, it was indicated that the steam explosion leads to condensation reactions of tannins and flavonoids, which become insoluble (Lomax et al., 1994).

Some researchers detected increased lignin yields after pretreatment, which were traced back to the formation of so called **"pseudo-lignin"** (Jakobsons et al., 1995; Li et al., 2009). For biorefineries, which target the production of furans based on the acid-catalyzed dehydration of C_6 and C_5 sugars the formation of humins can lead to reduced yields of levulinic acid as a follow-up product. This can be probably related to the polymerization of furan-like molecules like HMF and furfural to form humins as shown by van Zandvoort et al. (2013). Sumerskii et al. (2010) investigated the formation of humins from mono- and disaccharides under conditions similar to industrial processes for the hydrolysis of wood. In this case, humins were formed by polycondensation reactions in combination with electrophilic substitution to form cross-linked polymers. Hexoses react further over HMF forming ether or acetal bonds between rings and pentoses react over furfural forming C-C bonds between the rings. Similar process conditions and sugar degradation products are typical for the steam explosion process and different researcher emphasized the presence of carbohydrate based water insoluble humic matter next to condensed lignin in the so called "pseudo-lignin" (Aarum et al., 2018; Jakobsons et al., 1995; Li et al., 2009).

Overall, wood lignin undergoes various depolymerization, degradation and repolymerization reactions, which lead to a changed lignin composition. Additionally, different molecules like tannins probably show similar behavior during steam explosion. Further, the polymerization of furans originated from sugar degradation products form humins, which are often correlated with the term "pseudo-lignin". Therefore, the solid process stream likely doesn´t contain solely cellulose and lignin, next to some hemicellulose residues, but the lignin fraction likely contains a complex mixture of not-hydrolysable substances.

Lignin – interactions during enzymatic hydrolysis

It is well known that lignin interacts with enzymes and the structural features of the residual lignin in the solid process stream strongly effects the affinity between lignin and enzyme as well as the lignin - enzyme interaction mechanisms (Yuan et al., 2021).

In literature no consistency is detected on the effect of the **S/G ratio** (Yuan et al., 2021), but some authors found a correlation between the G-lignin content and the affinity for enzymes (Guo et al., 2014; Kim & Lee, 2018). Further, the type of **linkage** seemed to influence the lignin enzyme interactions, especially the reduction of ß - O – 4 linkages. This was attributed to an increased amount of phenolic hydroxyl groups, if the ß - O – 4 linkages were cleaved and additionally the recondensation reactions led to an increased hydrophobicity, which was also attractive for enzymes. However, the presence of ß - ß and ß - 5 linkages seemed to hamper the enzymatic conversion, too (Huang et al., 2016; Yoo et al., 2017; Yuan et al., 2021).

The **mechanisms** behind lignin - enzyme interaction are widely discussed and to the current state of knowledge are based on three mechanisms, which are steric hindrance, non-productive binding and deactivation. The mechanism of non-productive binding comprises three interaction aspects, the hydrophobic and electrostatic interaction as well as hydrogen bonding (Yuan et al., 2021).

Steric hindrance is one of the possible mechanisms. Pretreatments need to increase the accessibility of the substrate, but also lead to recondensation reactions and formation of "pseudo-lignin", which enhance steric hindrance. Other research found that hydrothermal treatment form lignin droplets, which reallocate at the substrate and thus hamper the accessibility for enzymes (Ko et al., 2015a; Ko et al., 2015b; Li et al., 2014; Wang et al., 2015).

Contrary to new findings, that the addition of specific lignin has a positive effect on enzymatic hydrolysis, the mechanism of **non-productive binding** is widely understood as the strongest factor to negatively influence enzymatic conversion. Additionally, during pretreatment the lignin

is modified, which possibly led to an increase in non-productive binding. The non-productive binding between lignin and enzymes were associated with hydrogen bonding as well as hydrophobic and electrostatic interactions (Yuan et al., 2021).

The **hydrophobic interaction** seemed to be the most important aspect of the non-productive binding mechanism. The hydrophobic part of the cellulases started to interact with the hydrophobic surfaces of the substrate. The strength of this interaction is dependent on the hydrophobicity of the enzyme, which is mainly influenced by the hydrophobic carbohydrate binding domain (CBM). This CBM contains different amino acid sequences and thus differ in their hydrophobic potential (Yuan et al., 2021). Depending on the lignin origin and modification, also the affinity for different enzymes is different. Lee et al. (2022) found that the CBM of cellobiohydrolases (CBHs) led to higher adsorption on oak lignin than on pine lignin and the endoglucanase Cel7B was two times more adsorbed by herbaceous plants than by wood lignin, which is contrary to the behavior of xylanase. Additionally, Guo et al. (2014) found the highest enzyme adsorption for CBHs and xylanases, whereas ß-glucosidase and endogluconase (EGs) were less adsorbed to the different tested lignins. Taking into account the lignin types the enzyme adsorption decreases in a row with pine > corn stover > aspen > kenaf. The different affinities of the enzymes towards the lignin were explained by different protein structures, as for example ß-glucosidases have no CBM (Guo et al., 2014).

Rahikainen et al. (2013b) investigated the interaction of the CBM structures of enzymes with lignin. It was indicated, that hydrophobic interactions take place between the CBM structure and lignin. The same aromatic amino acids were shown to contribute to the binding on cellulose and contrary also on lignin. Within an experiment with lignin films originated from steam exploded wheat straw and spruce, the spruce lignin showed the strongest and a fast adsorption of Cel7A of *T.reesei*, which further pointed out the raw material dependent affinity effects (Rahikainen et al., 2013b).

Electrostatic interactions represent the second aspect of non-productive binding mechanism. Due to the lignin modifications during pretreatment, the lignin contains specific amounts of functional groups like carboxyl, methoxyl or phenolic hydroxyl groups. During the enzymatic hydrolysis reaction, the lignin is thus negatively charged with pH values between 4.8 and 5.0. The charge of the enzymes mostly depend on its isoelectric point and the presence of functional groups like phosphate-, amino or carboxyl groups on the surface of the protein. Rahikainen et al. (2013a) tested lignin from spruce and straw after steam pretreatment and observed evidence for electrostatic interactions as the adsorption of enzymes decreased if the pH is elevated, likely due to electrostatic repulsion of the now negative loaded enzymes and lignin.

The interaction of phenolic hydroxyl groups with enzymes is associated with the formation of **hydrogen bonds** as the third aspect of non-productive binding mechanism. For instance, for the interaction of lignin of corn stover, kenaf and aspen a positive correlation between a higher phenolic hydroxyl group and a lower carboxyl group content is shown (Guo et al., 2014). Contrary, the presence of methoxyl groups seemed to have no influence on lignin-enzyme interactions and aliphatic hydroxyl- as well as carboxyl groups seemed to be advantageous for enzymatic digestion (Yuan et al., 2021).

The **deactivation** of cellulases by co-precipitation with phenolic compounds results in a loss of the initial protein structure and thus leads to irreversible deactivation (Yuan et al., 2021). During pretreatment different phenolics like vanillin or syringaldehyde are produced likely to be originated from lignin. For example, Tejirian and Xu (2011) as well as Ximenes et al. (2011) investigated the inhibitory and deactivating effect of different phenolic compounds. Some more details about the authors' research were already presented in the second part of chapter 2.12.3.

Next to the already presented mechanisms and influence of linkages and lignin types, there are further aspects, which have to be taken into account for the interpretation for lignin-enzyme interactions. These aspects are more related to the enzyme system itself.

The **composition** and **origin** of the **cellulase blend** has an influence on the affinity and sensitivity of enzymes towards lignin. For example, mono-enzyme systems behave differently than multi-enzyme systems (Yuan et al., 2021). Guo et al. (2014) worked with mono-enzyme systems to evaluate the enzyme adsorption on different lignin types. Contrary to that, Ko et al. (2015b) investigated the enzyme adsorption of a cellulase cocktail originated from *T. reesei* on liquid hot water pretreated hardwoods and found a different relation for the enzyme adsorption. It was shown that ß-glucosidases were the enzymes with the highest adsorption (Ko et al., 2015b). It was further shown, that the origin organism, which produced the enzymes played an important role, too. The ß- glucosidases of *A. niger* were less adsorbed than the ß-glucosidases produced by *T. reesei*. Additionally, the selective adsorption of enzymes from an enzyme cocktail resulted in indirect inhibitory effects, as the balance of co-working actors is hampered and probably lead to product inhibition (Ko et al., 2015b). Ximenes et al. (2011) also observed a lower deactivation of ß-glucosidases if originated from *A. niger* compared to *T. reesei*.

He et al. (2018) compared the interactions of **pseudo-lignin** and residual lignin with enzymes. The pseudo-lignin was generated by the pretreatment of monomeric sugars and holocellulose with dilute sulfuric acid and the residual lignin was originated from bamboo pretreated under acidic conditions. During these experiments, it was observed that the pseudo-lignin showed impact on cellulase activity due to non-productive adsorption. However, the impact on the cellulase activity was less severe for the pseudo-lignin than for the residual lignin from pretreated bamboo. These effects were indicated to differences in surface charges and hydrophobicity, next to others (He et al., 2018).

The interaction of lignin with enzymes during hydrolysis seem to comprise various interaction mechanisms and to be dependent on the lignin origin, comprising the type of biomass and pretreatment as well as on the enzyme system including composition and producing organism.

2.13 Influence of additives on enzymatic hydrolysis (EH)

The application of additives to enhance enzymatic conversion is frequently discussed in literature. The research was often directed to reduce the negative impact of residual lignin on the enzymatic hydrolysis for instance by non-productive binding of enzymes on lignin. Various surfactants like Tween, PEG or Triton were tested, as well as proteins like Bovine serum albumin (BSA) (Eriksson et al., 2002; Yang & Wyman, 2006; Yuan et al., 2021).

Different mechanisms were indicated to explain the effect of **surfactants**. One possible effect was that the surfactants change the substrate and increase the accessibility for the enzymes, for example by affecting the swelling of lignocelluloses. An increased availability of reaction sites for cellulases resulting in an increased conversion rate is another potential effect. Further, an improved protein stability due to surfactants is emphasized. Lastly, changes in the enzyme-substrate interactions during the presence of surfactants could potentially decrease non-productive binding effects (Ballesteros et al., 1998; Helle et al., 1993; Kaar & Holtzapple, 1998; Kurakake et al., 1994; Ooshima et al., 1986; Seo et al., 2011).

One of the potential main reason for the positive effect of surfactants is the disruption of the enzyme-substrate interactions due to the hydrophobic and hydrophilic molecule parts of the surfactant. The hydrophobic part of the surfactant interacts with the hydrophobic parts of the lignocellulose matrix and the hydrophilic part of the surfactant is unattractive for the enzyme, thus keeping it available in the solution. The especially positive effect of non-ionic surfactants

is explained by the presence of polyoxyethylene chains, which interact with the substrate by hydrogen bonds resulting in a strong occupation of the respective surface leading to steric interactions. This is also shown in researches about polyethylene glycol to surface interactions (Eriksson et al., 2002; Malmsten et al., 1998; Malmsten & Van Alstine, 1996; Sivars & Tjerneld, 2000).

Eriksson et al. (2002) tested different surfactants on steam exploded spruce wood and their effect on glucose conversion and enzyme adsorption. For the experiments mainly an enzyme load of 66 ml^{-1} FPU and a sample load of 50 g/l was used and the reaction ran for 24 h at a temperature of 40 °C and a pH of 4.8. Under these conditions, the non-ionic surfactants showed the biggest improvement in glucose conversion and reduction in enzyme adsorption. Additionally, the effect of Tween20 was stronger with reduced enzyme loadings, which enabled to half the enzyme load without reduced yield compared to the initial outputs. Experiments regarding the enzyme stability showed no impact of Tween20, but a slight increase in temperature stability (Eriksson et al., 2002).

It was indicated that the effect of Tween20 is focused on the solid-liquid interactions and that Tween20 showed no effect on liquid phase interaction, which would influence for example the ß-glucosidases (Seo et al., 2011). Experiments with de-lignified steam exploded spruce wood and microcrystalline cellulose (MCC) showed no reduced enzyme adsorption compared to the material containing lignin. Therefore, the effect of Tween20 seemed to be focused on the lignin (Eriksson et al., 2002). Especially for Tween20 a higher affinity towards lignin than towards cellulose was detected. The interaction of Tween20 happens over hydrogen bonding, thus one explanation for the increased affinity of the surfactant to lignin are the functional groups within the lignin. Carboxyl and phenolic hydroxyl groups can form stronger hydrogen bonds than polysaccharides, additionally, the hydroxyl groups of polysaccharides are partly included in inter and intra molecular hydrogen bonding and thus not available (Kaiser, 2018).

Controversial, Lee et al. (2021) found no notable improvement of enzymatic conversion of untreated rice straw if the surfactant Triton X-100 was added, but could proof that the surfactant adsorbs on the material surface. This emphasizes the assumption that the enzyme-substrate interaction in this case is highly influenced by the cellulose accessibility and less by non-productive binding of enzymes to lignin. Additionally, another study showed that the enzymatic conversion is increased after grounding of the material. If Tween20 is added to the grounded material this results in a higher conversion efficiency, especially for acid treated materials. Contrary, it seems to have less to no effect on material, which is alkali pretreated (Wang et al., 2020). Further, the effects of surfactants are indicated to be enzyme specific. For example Hsieh et al. (2015) observed a positive effect of PEG on CBH I during the hydrolysis of Avicell, but no effect on tested EGs and ß-glucosidase.

Next to the capabilities of surfactants to decrease non-productive binding, there is research about improved lignin extraction through surfactants. For instance, for a Kraft lignin model compounds, a dissolution of 100 % was detected with a Tween20 concentration of 0.5 mol/l. Further tests, of the capability of lignin extractions on sawdust of Marine pine showed positive effects for Tween20, if the concentration was kept low to prevent micelle formation, which decreased the lignin yield. Additionally, the lignin yield could be successfully increased, if cationic surfactants were applied (Melro et al., 2021).

Eriksson et al. (2002) also tested the **protein Bovine serum albumin (BSA),** which is known to adsorb on surfaces and found that the same increase in conversion can be reached with 17 g/l BSA compared to the effect with 2.5 g/l Tween20. However, the parallel addition of BSA and Tween20 shows no further beneficial effects. Also Yang and Wyman (2006) found an increased cellulose conversion from 54.2 % to 73.5 %, if BSA is added prior to the reaction, using an enzyme load of 20 FPU/g and SO_2 catalyzed steam exploded Douglas fir as substrate. Additionally, a possible reduction of the enzyme load without decreased conversion

is reported and these findings were in line to the assumption of a competitive binding mechanism of BSA to lignin, if cellulases are present (Yang & Wyman, 2006).

2.14 Influence of extractives on enzymatic hydrolysis

Next to the main components cellulose, lignin and hemicelluloses, lignocelluloses and especially wood contains the more or less small fraction of extractives. One big group of extractives known to interact with proteins are the tannins (Mai & Zhang, 2023). In relation to the orientation of this thesis, the following paragraphs focus mainly on the effects and mechanisms of **hydrolyzed tannins**. However, condensed tannins are also frequently studied about interactions with proteins (Hagerman, 1989).

For **individual substances**, which are part of the tannin group or known as individual wood extractives, no negative effect on enzymatic hydrolysis was detected for example for ellagic acid and flavonol, at least at low concentrations. However, epicatechin as a flavonoid showed a strong hampering effect even at low concentrations (Mai & Zhang, 2023; Tejirian & Xu, 2011). Additionally, the sensitivity of the enzymes seemed to depend on the **producing organism**. For instance, gallic acid showed small deactivation rates of ß-glucosidase if it was originated from *A. niger*, but a deactivation rate of > 60 % for ß-glucosidases from *T. reesei (Ximenes et al., 2011)*.

Tannins comprising **several galloyl groups** like tannic acid decreased the yield of enzymatic hydrolysis already at low concentrations about 70 - 80 % (Tejirian & Xu, 2011). This was in line by results of another study, which showed a decrease of 60 % for the production of cellobiose and a complete stop of glucose formation if tannic acid was present during the hydrolysis of filter paper. The inhibiting effect was directly detected from the beginning of the reaction (Ximenes et al., 2011). Interestingly, the effect was stronger for ß-glucosidase from *T. reesei* and notable lower if it was originated from *A. niger,* which again points out the effect of enzyme origin. Next to inhibition effects, also a strong deactivation of ß-glucosidase in *T. reesei* and *A. niger* was detected with values > 70 % for tannic acid (Ximenes et al., 2011).

Goldstein and Swain (1965) found that between ß-glucosidase from sweet almond and tannic acid a factor of 1:0.46 forms, regarding the binding reaction balance. It was further determined, that the enzymes bound by the tannins were still active and by addition of the non-ionic detergent Tween80 the tannin - enzyme complexation was completely reversed. Additional experiments with PEG with an average size of 20,000 resulted in good re-activation results. Based on these findings, it is emphasized that hydrophobic interactions as well as hydrogen bonding take place within tannin and protein interactions, leading to the formation of protein – tannin complexes, which precipitate (Goldstein & Swain, 1965; Gustavson, 1954; Oh et al., 1980).

It seems that the **molecular size** of the tannin, as well as its flexibility and mobility are important properties to evaluate the protein binding capacity and affinity. For example, the molecular size seemed to influence the protein binding capacity strongly in case of galloylglucose derivatives, as each additional galloyl group increased the adsorption capacity. However, after the formation of depsides and oxidative coupling reactions, like in gallo- and ellagitannins, the importance of molecular size in regard to the binding affinity decreased and the flexibility and mobility of the structure became more important (McManus et al., 1985). Further, the interactions of tannins and proteins was indicated to be dependent on the pH, which seemed to be linked with the isoelectric point of the respective protein (McManus et al., 1985).

For the interaction of tannins with proteins, two different scenarios were described. In the first scenario, the tannins bind to the protein, occupy the surface and thus increase the hydrophobic properties, which lead to aggregation between similar particles and followed by precipitation.

The second scenario additional includes the cross-linking between different proteins by tannins. Whereas the first scenario is described for low protein concentrations, the second scenario seems takes place at high protein concentrations (McManus et al., 1985).

Regarding the **hydrophobic interactions** of tannins, it was found that an increase in galloyl groups decreased the solubility of gallotannins. However, the solubility could be increased if amino acids were present. This effect was assigned to the aliphatic side chains of amino acids, which interacted with the hydrophobic tannin parts, whereas the carboxylic and amino groups of the amino acids interacted with water and thus increased the solubility. Therefore, there was indication that tannins interact with hydrophobic parts of the proteins, which are build out of amino acids. Further, it was indicated that tannin-protein complexation does not necessary lead to precipitation, but is dependent on the solubility of the formed protein - tannin complex (He et al., 2006).

However, the molecular size of tannins as a scale for its hydrophobicity was shown to be not the sole way of interaction. The **phenolic hydroxyl groups** of tannins and the polar groups of proteins seem to have a strong influence, too. This conclusion explained the stronger binding affinity of the protein BSA then for instance pepsin towards pentagalloylglucose. It was further shown, that gallotannins also interact with sugars and polysaccharides by hydrogen bonding of the hydroxyl groups of the saccharides and gallotannins. However, the interactions seemed to be stronger between gallotannins and proteins or phospholipids than saccharides (He et al., 2006).

Kawamoto et al. (1997) also found indication for irreversible denaturation of proteins like BSA during interaction with galloylglucose. It is indicated that the precipitation increases if the numbers of galloyl groups is increasing. Additionally, the dimensional order of the galloyl groups play a role, which are more reactive in positions with lowest hindrance to each other (Kawamoto et al., 1997). Additionally, it was repeatedly found that the peptide amide group of the enzymes bonds with the phenolic hydroxyl groups in tannins (Kawamoto et al., 1997).

Overall, the interaction of tannins and enzymes is a complex matter, which seems to be dependent on the molecular structure of the tannin and the protein, which controls the general affinity for complexation. Further, the producing organism and pH seem to play a role in the interaction processes.

2.15 Bark in context with SE pretreatment and enzymatic hydrolysis

Considering the particular features of wood bark, some studies investigated the influence of bark on the steam explosion process and further processing. For example, Robinson et al. (2002) investigated the fermentation of Douglas fir to ethanol by adding varying bark proportions to the SO_2-catalyzed steam explosion pretreatment. The glucan content of the initial wood was ~ 46 % and of the bark ~ 15 %. Contrary, the proportions of **acid insoluble lignin** and extracts were higher in bark than in wood. The composition after SE was found to be dependent on the bark content of the batch. The sugar content decreased with rising bark proportion, but at the same time, the share of the solid residue increased as well. Interestingly, it was observed that the content of **sugar degradation products** like HMF and furfural decreased slightly with increasing bark proportion, but the content of lipophilic compounds increased. Except the lower sugar content due to bark, no negative impact on ethanol conversion was detected up to a bark proportion of 30 % (Robinson et al., 2002).

Similar to Robinson et al. (2002), Frankó et al. (2015) detected an increased proportion of acid insoluble lignin after SO_2-catalyzed steam pretreatment of spruce with variable bark proportion. Additionally, a neutralizing effect of bark on the pH during pretreatment is assumed as well (Frankó et al., 2015). For the utilization of softwood bark of Norway spruce and Scots pine a

hot water extraction prior to acidic catalyzed steam explosion, which removed a high proportion of the water soluble extractives, increased the sugar conversion during subsequent EH and reduced the proportion of acid insoluble lignin. It was assumed that the removed fraction of water soluble extractives, could not react towards pseudo-lignin, resulting in the detected decrease in insoluble lignin. (Frankó et al., 2018).

Boussaid et al. (2001) tested chipped White fir and Ponderosa pine material from a thinning harvest, with an estimated bark content of 9 % for fermentation after SO_2 catalyzed steam explosion pretreatment. Interestingly, a dependence of the fermentability to the severity is detected, which showed no negative impact of bark for material processed with high severity. However, at the same time a decreased ethanol yield ~ 67 % (compared to ~ 87 % if no bark is present) is detected at lower severities and this is even the case, as samples without bark showed the highest inhibitor concentration. These findings were explained by a potentially stronger condensation of polyphenolic substances in bark, e.g. tannins during higher severities, thus showing less inhibition during fermentation (Boussaid et al., 2001). Another study also assumed a changing lignin to tannin ratio dependent to the steam explosion pretreatment severity with temperatures of 189 °C and 218 °C and an increase of the molecular weight of the polyphenolic material (Lomax et al., 1994).

Frankó et al. (2015) analyzed the **liquid fraction** after SO_2-catalyzed steam pretreatment of spruce wood chips with different proportion of bark. The glucose content decreased from ~ 27 g/l towards ~ 18 g/l if the bark content was increased from 0 - 50 %. This effect was explained by the lower polysaccharide content of bark. Without the presence of bark, values around 2 g/l were detected for furans and formic or acetic acid. Additionally, levulinic acid was detected. Those values strongly decreased if bark was included in the material (Frankó et al., 2015).

Overall, the different chemical composition of bark compared to wood, seem to influence the pretreatment process and subsequent enzymatic hydrolysis, in terms of sugar content and proportions of insoluble lignin and sugar degradation products.

2.16 Different aspects of minerals during biomass conversion

For biomass combustion or thermochemical conversion processes like pyrolysis, biomass ashes (minerals) play an important role, as these are known to cause fouling and corrosion in the machineries (Brown, 2019). The recycling of minerals as fertilizers or applications for construction materials like cement or concrete is discussed in literature. At the same time, problems arise by the accumulation of heavy metals and the high variability of the mineral composition due to the biomass type and influence of the biomass origin, and harvesting conditions. The disposal and management of residual minerals as well as the maintenance cost for the machinery are economic factors, increasing the operating costs (Kenney et al., 2013; Vassilev et al., 2013; Zając et al., 2018).

In contrast to the thermochemical conversion of biomass, there is no direct accumulation of mineral residues during conversion processes like steam explosion and enzymatic hydrolysis, as the biomass is permeated by an aqueous solution or steam. However, it is likely that the process conditions and the type of raw material strongly influence the distribution of the minerals within the different process streams.

Werkelin et al. (2010) investigated the leaching of different woody materials during a three step washing process using water, followed by a NH_4Ac buffer and a aqueous HCl solution. The leaching with water strongly decreased the K and Na content, but some proportions of Mg, Mn and Ca were removed as well. The buffer leaching mainly removed Mg, Mn and Ca. The acidic leaching removed proportions of Fe and Al. The solubility within the media seemed to correlate with the valence of the ions. Interestingly, it was shown that for wood bark samples the main Ca fraction was leached during the acidic leaching conditions. The non-metal minerals Si and S were found to mainly stay insoluble, but Cl and P could be removed strongly by leaching with water. Based on the results of the authors work it is indicated that the Cl and proportions of P and S are present as potassium salts (KCl, KH_2PO_4, K_2SO_4) and that the main Ca proportion found in bark is associated with calcium oxalate minerals. Further notable proportion of the P and S seem to be covalently bound to the organic matrix (Werkelin et al., 2010). Further, Aston et al. (2016) showed that acidic washing of corn stover effectively removed alkali metals and alkaline earth metals as well as chlorine and phosphor.

Next to the distribution of the minerals within the process streams due to their solubility and the respective process conditions, minerals seem to have a direct influence on processes like enzymatic hydrolysis and fermentation. Hörhammer et al. (2018) found an increase in monomeric sugar yield for short rotation poplar, washed under different condition prior to steam explosion and enzymatic hydrolysis or fermentation. However, it could not be clearly shown if this is solely due to the decrease of mineral content or also through extraction of sugar and lignin degradation products. The glucose conversion is found to be 62.4 % for untreated poplar and 68.3 %, 74.1 % or 76.0 % if it was pretreated by neutral, acidic - neutral and acidic washing prior to steam explosion. Additionally, the buffering capacity of the woody material is found to be lower for acidic - neutral or acidic washed material, due to the lower mineral content. Next to Hörhammer et al. (2018) also Le et al. (2014) found a pH dependency on the amount of leached minerals during hydrothermal pretreatments from wheat straw. Further, a correlation of the decreasing elements like P, Mg, K, Mn and Ca with the residual presence of hemicelluloses in the solid fraction after pretreatment was detected (Le et al., 2014).

de Mello et al. (2022) described in his review the effect of impurities on sugar cane bagasse processing as well as some research about enzyme inhibition by $Ca^{2+,}$ K^+ and Mg^{2+} ions on invertase during fermentation. In contrast, Akimkulova et al. (2016) showed a positive effect of $MgCl_2$ salt on enzymatic conversion, due to a reduction of non-productive binding of enzymes to lignin. This is explained by the interaction of the Mg^{2+} ions with the phenolic OH groups of lignin. Additionally, Liu et al. (2010) found a positive effect of Mg^{2+} and Ca^{2+}, but a negative one for Cu^{2+} and Fe^{3+} during enzymatic hydrolysis and the presence of lignin.

Following these research outputs, there is a need to monitor the mineral leaching during the biorefinery processes including the main process stream and side streams. On the one hand, this is important to understand potential interactions in following processes like enzymatic hydrolysis or fermentation. On the other hand, the knowledge about the accumulation of specific mineral fractions within different process streams is important to adapt the handling for example in case of waste water management or machinery maintenance. Further it possibly opens up further utilization potentials of side process streams, for instance by extracting plant nutrients for fertilizers.

3. Thesis frame and research questions

Specific challenges within the current development towards a sustainable, resistant and resilient economic system are to establish circular utilization routes based on renewable raw materials, including flexible and thus reliable productions and supply chains. The aim is to reduce the dependency on fossil fuels. To enable this development it is necessary to use the available renewable raw material portfolio in the most extensive way possible, without exploitation and destabilization of the ecosystem (BMBF & BMEL, 2020; BÖR, 2023).

The implementation of biorefineries to use the individual biomass components is one promising step to approach these challenges (BÖR, 2023; FNR, 2012). Wood is one of the best available renewable raw materials without competition to food valorization (Dahmen et al., 2019). Additionally, wood contains high amounts of valuable components like cellulose, lignin, hemicelluloses and extractives (Mai & Zhang, 2023).The high complexity of the wood matrix is in general challenging for conversion processes in biorefineries. Additionally, the characteristics like chemical composition or structure of the wood tissue are partly species-specific, which increases the complexity of raw material - process interactions (Berg & Guzmán, 2023; Shafiei et al., 2015).

The adaptation of German forests towards climate stable and resilient forest stands implement a higher hardwood volume and a diverse species portfolio. Additionally, an increased proportion of extractive rich tree species like oak becomes likely, due to the changing growing conditions (BMEL, 2021; Klemmt et al., 2018). However, especially extractive rich species are challenging for conversion processes, like it is known for the pulping processes (Ek et al., 2007).The shift within the raw material portfolio and the need to prioritize material over energy use (BMBF & BMEL, 2020) clearly puts the focus on the utilization of hardwoods.

Therefore, from the biorefinery perspective, the frame of this thesis was set on the processes of the first refining step. This included the pretreatment of the raw material (wood chips) by steam explosion under acidic conditions, resulting in a solid process stream (SPS) and a liquid process stream (LPS). Further, it included the conversion of the SPS by enzymatic hydrolysis (EH) towards the main platform glucose. The focus on the first refining step was based on the assumption, that potential species-specific influences were most likely visible during the processes of the primary refining. The vapor fraction was excluded due to the high dependency on machine aspects, regarding kinetics and location of condensation as well as in regard to impurities and possible losses.

To include the aspects of tree species variability and species-specific characteristics, four tree species were chosen: beech *(Fagus sylvatica)*, ash *(Fraxinus excelsior)*, oak (*Quercus robur/petraea)* and chestnut *(Castanea sativa)*. Additionally, a mixed batch (60 % beech, 20 % ash and 20 % oak) was comprised. These species were chosen based on their capacity to ensure a sufficient supply for an industrial plant (beech) or the availability from an economic perspective (ash). Oak and chestnut were chosen because of their predicted good climate stability. Additionally, these species are not regularly used in this kind of applications so far, which is likely reasoned in their high extractive content (Ek et al., 2007).

It is known that the effects of pretreatment and enzymatic hydrolysis are dependent on the type of biomass. However, a deepened investigation of species-specific effects within one type of biomass and the parallel investigation of suitability and additional potentials is missing so far or just partly included (Cotana et al., 2015; Grous et al., 1986; Schultz et al., 1984). To start at this point the first approach centered species-specific influences on the composition of the raw material and the distribution of the components within the process streams. In detail, the proportions of cellulose, hemicelluloses, lignin as well as extractives, sugar and lignin degradation products as well as minerals were comprised. Additionally, the evaluation of the suitability regarding the yield of the main target products and additional valuable products was

included within this first approach. This resulted in the first research area of this thesis involving three individual research questions (Rq).

1. The first research area worked on the usability, including suitability and potentials of the different tree species as raw material for biorefinery processes, which target the utilization of the individual wood components.

1.1. How to evaluate the suitability of the different tree species for the production of the main platform glucose by enzymatic hydrolysis?

1.2. Which species-specific patterns are detectable in the liquid and solid process stream?

1.3. How does the pretreatment process distribute the different components within the process streams?

To date, the future distribution of the available wood assortments between the different actors in wood industry is not clear, but likely hardwood energy wood assortments, will be future raw material sources for lignocelluloses biorefineries. As these assortments often include the utilization of bark as additional energy source, the utilization of bark as raw material in biorefineries becomes interesting as well. The processing of wood and bark could contribute to the overall yield. Further, the debarking process is an economical aspect, which needs to be considered. The presence of bark in the conversion processes is a new factor, because bark has a different chemical composition than wood. There is just little research available, which investigates the suitability of wood - bark mixtures for the SE process and enzymatic hydrolysis. Further, the available studies focus mainly on softwood barks (Boussaid et al., 2001; Robinson et al., 2002).

To target this gap, two scenarios were included in this thesis. The first scenario used wood chips of beech, ash, oak and chestnut originated from stems, which were debarked (db) prior to the chipping. The second scenario includes the same species, but the wood chips were originated from stems, which were not debarked prior to chipping. The wood chips with bark (wb) thus represented a wood - bark mixture, considering the natural bark proportion of standing trees. This aspect led to the second research area including three individual research questions:

2. **The second research area focused on the influence of bark on the processing of the material during pretreatment and enzymatic hydrolysis in terms of profitability and additional potentials**.

2.1. How does the presence of bark influence the suitability of the tree species for the production of the main platform glucose by enzymatic hydrolysis?

2.2. How does the presence of bark influence the composition of the different process streams?

2.3. Which effect does the presence of bark have on the factors influencing enzymatic hydrolysis?

The research about material disintegration during steam explosion and followed enzymatic hydrolysis, revealed a high variety in possible factors influencing the conversion. Most works focus on a specific factor, its mechanism or changes by variations within the process conditions. However, the evaluation of the impact severity of the factors in relation to tree species-specific differences are not well known. Further, the knowledge about potentially interconnections of species-specific characteristics with effects on enzymatic hydrolysis, the underling mechanisms as well as options to equalize these effects would be highly valuable instruments. These considerations led to the third research area of this thesis, comprising three research questions:

3. The third research area dealt with different factors influencing enzymatic hydrolysis and the detectable variations related to tree species-specific effects.

3.1. *How to evaluate influencing factors in terms of impact on enzymatic hydrolysis?*

3.2. *How are the tree species-specific characteristics interconnected with influencing factors on enzymatic hydrolysis?*

3.3. *Which options are available to deal with potential species-specific effects during enzymatic hydrolysis?*

For a better overview on the thesis frame, the included processes, used samples and scenarios are visualized in Figure 4. The laboratory work included the analysis of the raw materials and the SPS as well as the LPS. Additionally, the EH were performed individually in the laboratories. Solely, the pretreatment was performed externally, by an industrial partner.

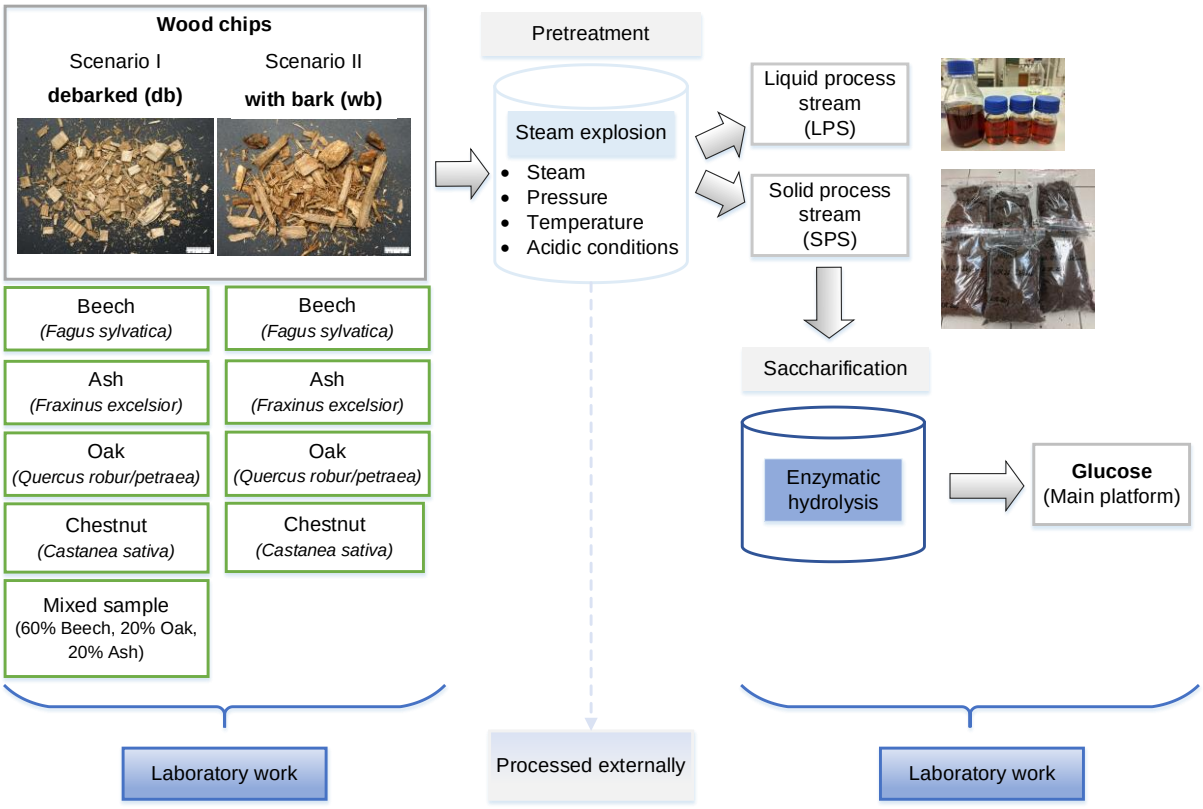


Figure 4: Thesis frame and sample materials

4. Material and methods

This chapter is subdivided into three sections. Chapter 4.1 describes the used materials and the main sample preparations. Chapter 4.2 presents the methods used to characterize and analyze the materials and chapter 4.3 presents detailed information about the instrumental analytics.

4.1 Materials

In the following detailed information about the different materials used in this thesis and the sample preparation steps applied for the wet chemical analyses are given. Additionally, Table 2 contains an overview on the tree species and different sample batches as well as used abbreviations for the sample naming.

4.1.1 Raw material and sample preparation

For the starting materials **wood chips** of European beech *(Fagus sylvatica)*, Common ash *(Fraxinus excelsior)*, English/Sessile oak *(Quercus robur/petraea)* and Sweet Chestnut *(Castanea sativa)* were used. The tree stems originate from forest stands in northern Germany. Beech, oak and ash trees were harvested in the forest stands of the “Herzoglich Oldenburgische Verwaltung”. The chestnut stems were harvested in the forest stands of the “Schleswig-Holsteinische Landesforsten”.

According to the harvesting lists, the accounted stem sections showed average diameters (without bark) of 44 cm for beech and oak stems and 33 cm for ash stems. The chestnut stems had an average diameter (without bark) of 20 cm. In total 6.7 m^3 beech, 6.6 m^3 ash as well as 6.8 m^3 oak and 11.2 m^3 chestnut were harvested.

From each of this batches, two sub batches were formed, which were treated separately following scenario I and scenario II. For scenario I the stems were chipped in a drum chipper with prior debarking. This sub batch contained **debarked (db)** wood chips. For scenario II the debarking step (drum chipper) was not performed, thus these stem sections were chipped containing their natural bark proportion. This sub batch contained wood chips **with bark (wb)**.

For each scenario four sample batches containing single-origin wood chips form beech, ash, oak or chestnut were available. Additionally, a mixed sample batch containing 60 % beech, 20 % ash and 20 % oak chips was included. The mixed sample batch was prepared using debarked material. The harvesting and processing of the wood chips was organized by the project partner. The wood chips were delivered non-screened in fresh state.

For the **chemical analysis**, the fresh wood chips were air dried (dry matter > 90 %) and afterwards milled using a cross hammer mill from Retsch with a 0.75 mm sieve. The milling process was cooled by dry ice. The wood powder was sieved in a sieving tower (Retsch AS 200) with an amplitude of 0.8 mm/”g” for 10 min. Using a 0.315 mm and a 0.05 mm sieve and three sieving balls in the biggest sieve. The fractions > 0.05 < 0.75 mm were used for further analyses.

4.1.2 Solid process stream (SPS) and sample preparation

The prior described wood chip batches (db and wb) were further processed by the industrial partner in pilot scale by steam explosion under acidic conditions.

The resulting solid process stream (SPS) prior to washing was referred to as SPSpw and was solely used for the analyses described in chapter 5.11. For all other analyses the SPS after washing was used and is thus referred to as SPS.

For the wet chemical analyses the air dried SPS (dry matter > 90 %) was sieved in the same sieving tower than the wood powder, using a 1.00 mm sieve with three sieving balls and an amplitude of 1.5 mm/"g" for 10 min. The fraction < 1.00 mm was used for the analyses.

4.1.3 Liquid process stream (LPS) and sample preparation

The LPS resulted from the SE process and additionally contained the washing water of the SPS already mentioned in the previous chapter. The aqueous process stream comprised a precipitating fraction, which was removed by filtration under vacuum using a 0.45 µm PES filter (hydrophilic, Pall Corporation) prior to further analyses.

Table 2: Overview on the included tree species and samples as well as respective abbreviations.

Tree species			**Abbreviations**
European Beech	*(Fagus sylvatica L.)*		Beech
Common Ash	*(Fraxinus excelsior L.)*		Ash
Englisch/Sessil Oak	*(Quercus robur L./petraea Liebl.)*		Oak
Sweet Chestnut	*(Castanea sativa Mill.)*		Chestnut or Chest
Raw material - Wood chips			
Beech Ash Oak Chestnut		*db (=debarked)*	wb (=with bark)
Mixed sample	(60% Beech, 20% Ash, 20% Oak)		
Solid process stream prior to washing			**SPSpw**
Beech Ash Oak Chestnut		*db (=debarked)*	wb (=with bark)
Mixed sample	(60% Beech, 20% Ash, 20% Oak)		
Solid process stream			**SPS**
Beech Ash Oak Chestnut		*db (=debarked)*	wb (=with bark)
Mixed sample	(60% Beech, 20% Ash, 20% Oak)		
Liquid process stream			**LPS**
Beech Ash Oak Chestnut		*db (=debarked)*	wb (=with bark)
Mixed sample	(60% Beech, 20% Ash, 20% Oak)		

4.2 Material characterization and analysis

This chapter contains the methodical description of the individual analyses applied to characterize and analyze the materials. The analyses ran in double, if not stated otherwise.

4.2.1 Determination of solid matter

Vacuum oven 40 °C

The material was weight into constant weighing glasses and dried in a vacuum oven from Heraeus (RVT 360) at 40 °C and a vacuum of 16 bar overnight for 16 h, with silica gel as drying agent (Carl Roth, Karlsruhe, Germany). The samples were cooled for 30 min in a desiccator and weighted to constant. Experiment was performed in double. If the material was very wet, it was pre-dried under the same conditions during the day.

Oven at 105 °C

The material was weight into constant weighing glasses and dried at 105 °C to constant. The oven was from Memmert (UI 30). Experiment was performed in double. This method was solely used for the dry content determination of the successive extracted wood powder and quantification of AISR after acid hydrolysis of wood and the SPS

Freeze drying

For the determination of the solid matter of the liquid process stream or the hot water extracts freeze drying was applied with a device from Christ, Alpha 1-2 LDplus and the samples were weighted to constant afterwards.

4.2.2 Incineration

For the determination of the mineral content 1 g of sieved wood powder or the SPS was smoldered till no more smoke was released. The sample was further incinerated for 2 h in a muffle oven from Heraeus MR 170 at a temperature of 525 °C. The sample was weighted to constant in a 1 h re-incineration and 1 h cooling cycle. The method was based on Tappi instructions about “Ash in wood, pulp, paper and paperboard: combustion at 525 °C” (TAPPI, 1993).

4.2.3 Extractions and pH measurement

Solid phase extraction (SPE)

In some cases, a SPE was applied for sample preparation prior to HPLC or GC/MS analyses. For the SPE cartridges from Waters (WAT 020515 *big and WAT 023501 *small) or Chromafix C18 ec cartridges size M from Macherey – Nagel were used. The cartridges were first pre-conditioned by filtering 5 ml of MeOH (Suprasolv from Merck) followed by 10 ml of ultrapure water. Subsequent, the sample was slowly filtered through the cartridge. The first 3 ml of the extracted samples were discharged and the residual amount collected as “extracted phase”. Afterwards the cartridge was flashed with 10 ml of ultrapure water. The adsorbed material was eluted by 10 ml of acetonitrile : water (1 : 1) and 5 ml of pure acetonitrile (ACN) (VWR for HPLC). The eluted phase was called “eluate”.

Hot water (HW) extraction of wood

For this experiment 10 g of air dried and sieved wood powder was mixed with 500 ml of deionized water and 4-5 boiling stones were added. The sample was boiled under reflux for 3 h. After that, the liquid was decanted into glass filter crucibles with a porosity of 3 (16 - 40 µm).

The extracted wood powder was washed with hot deionized water and air dried. For the quantification of the extract content, an aliquot (100 ml) was taken from the decanted liquid, frozen, freeze-dried and weighted to constant. Extractions were performed in double. For the samples oak and chestnut db and wb as well as the mixed sample, the wood powder was extracted two times. After the first 3 h the liquid was decanted and replaced by fresh deionized water. The sample was further extracted for 3 h.

Further qualification of the hot water extracts was done by GC/MS analyses (see chapter 4.2.5 and chapter 4.3.5)

pH measurement

The pH of the hot water extract was measured in the decanted extract liquid after 3 h extraction and cooling to room temperature. The pH measurement were performed in double with a calibration between pH 4 (Buffer solution from VWR) and pH 7 (Buffer solution from Carl Roth). For the measurement, a pH device QpH70 from VWR was used including temperature correction. The pH meter was equipped with a Mettler Toledo InLab Science electrode.

Soxhlet extraction with organic solvents (successively) of wood

The extraction was performed successively on wood powder with the organic solvents petrol ether > acetone > methanol using a Soxleth extractor. The solvents methanol and acetone were SupraSolv-quality and were purchased by Merck. The petroleum ether (for pesticide residual analysis) was purchased from VWR. 5 g of air dried, milled and sieved wood powder was weighted in cellulose extraction tubes and covered with plated filters. 170 ml of solvent and four boiling stones were added and the sample was extracted in double for 6 h. The extract was evaporated at a rotary evaporator at 25 °C and weighted to constant. The extracted wood powder was air dried and used for the next extraction with the next solvent in the row. The dry content was determined after each extraction step using the 105 °C oven method. For the samples, oak and chestnut wb the extraction time during the methanol extraction was extended by 3 h to ensure a sufficient extraction.

Further, qualification of the solvent extracts was done by GC/MS analyses following Method A (see chapter 4.3.5)

Acetone / H_2O extraction of the SPS

The extraction was performed on air dried SPS and wood powder. For the extraction of the dried material a pre-extraction with petrol ether using a Soxhlet apparatus was performed for 6 h. For the successive extraction with acetone / water (Ace/H_2O) (7:3) the solvent was directly added to the material using a solid to solvent ratio of 1:15. The sample was extracted under continuous stirring at ambient temperature for 24 h. Experiments ran in double. Afterwards the sample was filtered using glass filter crucibles (10 – 16 µm). The petroleum ether (for pesticide residual analysis) was purchased from VWR and the acetone was from Merck in SupraSolv-quality. The petroleum ether and the Ace/H_2O extracts were dried and quantified gravimetrically.

The Ace/H_2O extract was further qualitatively analyzed by FTIR as well as pyrolysis GC/MS measurements applying Method D and E (see chapter 4.3.5). For the quantification and qualification by GC/MS measurements (Method B), reference substances were used to develop an external calibration, similar to the procedure explained in chapter 4.2.5 (The Liquid process stream (LPS) after SPE)

4.2.4 Acid hydrolysis

The applied method was based on the NREL instructions about “Determination of Structural Carbohydrates and Lignin in Biomass” (NREL, 2008). All experiments were performed in double.

Around 300 mg of dry, hot water extracted wood powder or dry SPS was weighted and mixed with 3 ml of sulfuric acid (72 %) (98 % for analysis, Merck). The sample reacted for 1 h in a water bath at 30 °C and was mixed in between, followed by dilution with 84 ml deionized water. The samples were autoclaved at 121 °C for 60 min (HV – 85L, HMC Europe).

After cooling, the sample was filtered through porcelain filter crucibles. The filtrated hydrolysate was used to determine the sugars and the acid soluble lignin. The solid residue was used to determine the acid insoluble lignin gravimetrically. The acid soluble or insoluble lignin was referred to as acid soluble residue (ASR) and acid insoluble residue (AISR) within this thesis. The acid insoluble residue was washed with water until pH reached 5 – 6 and dried at 105 °C (chapter 4.2.1). The content was determined gravimetrically. The sugars were determined by HPLC measurements (chapter 4.3.2). Results were calculated and presented as anhydrous, without correction factors. To transfer monomers to anhydrous sugars the factor 0.88 was used for C_5 sugars and 0.9 for C_6 sugars, based on NREL (2008). The ASR was measured by diluting the hydrolysate and analyzing it by UV measurements (chapter 4.3.4).

4.2.5 Sample preparation for further composition analyses at GC/MS

Hot water extracts from wood

For the qualification of the hot water extracts by GC/MS analyses 10 mg dry HW extract was solved in deionized water (2 mg/ml) and separated by SPE, as described in chapter 4.2.3. The eluate was evaporated and weighted to constant. The resulting dry eluate was used for the GC/MS analysis following Method A (see chapter 4.3.5). SPE extractions were performed in double for each sample and GC/MS analyses are also done in double with two injections each.

The Liquid process stream (LPS) after SPE

An aliquot of 3 ml was taken from the respective LPS sample and diluted (1 : 3.5). This sample was separated by a solid phase extraction using a C18 Phase (see chapter 4.2.3). For GC/MS analyses, the procedure of Method B (chapter4.3.5) was used.

The quantification was performed by external calibration (4 points) for three individual substances: syringic acid (98 %, Carl Roth) ellagic acid (≥ 96.0 %, HPCE grade, Sigma-Aldrich) and secoisolariciresinol (≥ 95 %, HPLC grade, Sigma-Aldrich). Each calibration also contained heneicosanoic acid (> 98 %, Sigma-Aldrich) as internal standard. The calibration was based on the area ratio of the individual substances with the internal standard and the known substance concentrations. By applying a linear fit the linear calibration equation (y = a + x * b) was determined with the substance specific intercepts (a) and slopes (b).

All samples were mixed with the same internal standard prior to analysis at GC/MS. By using the peak area ratio of each substance with the internal standard, as the known variable (x), the substance concentrations were calculated (y). Solid phase extraction and GC/MS analyses were performed in double.

Liquid process stream (LPS) after DCM extraction

5 ml of the respective LPS sample were supplemented by an internal standard (heneicosanoic acid, > 98 %, Sigma-Aldrich), solved in DCM. The sample was 3 times liquid / liquid extracted with DCM (Supelco for gas chromatography, Merck). The DCM phase was dried over magnesium sulfate and gently evaporated to constrict the sample. Analysis at the GC/MS was performed as described in Method C (chapter 4.3.5).

Supernatants after enzymatic hydrolysis

An aliquot of the supernatant after enzymatic hydrolysis was filtered through a 0.45 µm PES filter (hydrophilic, Pall Corporation, Ann Arbor, MI, United States) under vacuum. From the filtered samples, 5 ml were taken to perform a SPE as described in chapter 4.2.3. The eluate was then prepared as described in Method A (chapter 4.3.5), but the measurement conditions of Method B (chapter 4.3.5) were applied to analyze the samples at the GC/MS. For quantification of the ellagic acid, an external calibration curve with ellagic acid was established.

4.2.6 Determination of acetyl groups

The quantity of acetyl groups in non-extracted wood powder was determined by an aminolysis reaction. The acetyl groups in the sample were cleaved by pyrrolidin (Fluka/Honeywell) and form acetylpyrrolidin, which was further quantified at the GC using propionylpyrrolidin as internal standard. The method was based on Masson et al. (1994). 30 mg of the sample was weighted into vials and pyrrolidin was added. For the reaction, the vials were placed in a thermoblock at 80 °C for 18 h and further analyzed at the GC using Method F.

First, the purity of acetylpyrrolidin and propionylpyrrolidin was reviewed. The substances were diluted by pyridine from Sigma-Aldrich and the ratio of substance and impurity peak areas were evaluated after measuring at the GC. Next, the internal standard solution was prepared by adding pyridine to the propionylpyrrolidin to get the respective concentration. To determine the response factor, acetylpyrrolidin was mixed with pyrrolidin and internal standard solution and analyzed at the GC using Method F.

4.2.7 Determination of S- and G-lignin proportions

For the determination of the guaiacyl- and syringyl- unit proportion a thioacidolysis was performed. Analyses ran in double. The method was based on the literature of Lapierre et al. (1986) and Rolando et al. (1992).

For sample preparation 40 - 60 mg of the SPS were transferred to a laboratory bottle and mixed with the reaction solution of boron trifluoride etherate, ethanthiol both from Merck and dioxane (Supelco for analysis, Merck). After flushing with nitrogen, the reaction took place at 100 °C in a thermoblock for 4 h.

For the extraction, a sodium hydrogen carbonate (99.5 %, for analysis, Merck) solution, DCM (Supelco for gas chromatography, Merck) and the internal standard solution with tetracosane (99 % for GC, Fluka/Honeywell) in dichloromethane were mixed and the cooled, prepared

sample was added. The aqueous phase was three times liquid / liquid extracted with DCM. The organic phase was dried over magnesium sulfate (VWR) and evaporated to constrict the sample volume.

For the quantitative analysis at the GC (Method G) an aliquot of the dried sample was mixed with pyridine and the derivatization reagent BSTFA, which is described in chapter *4.3.6.* For peak identification, the samples were analyzed at the GC/MS using Method A.

4.2.8 Characterization of enzyme solution and residual free protein in the supernatant

For all experiments with enzymatic hydrolyses the cellulase, enzyme blend from Sigma-Aldrich, containing the Cellic®Ctec2 (Lot# SLCG4389) substrate from Novozymes was applied**.** The enzyme solution was characterized by total nitrogen analysis at a total organic carbon analyzer (TOC-L) from Shimadzu. For the evaluation, a calibration curve based on the protein Bovine Serum Albumin (BSA) protease free (VWR) was used. The measurements resulted in a total protein concentration of 163,6 mg/ml.

Additionally, the enzyme activity was determined by Filter Paper Unit (FPU), based on the description of Ghose (1987) and NREL (1996), but with some adaptations. A 50 mM sodium acetate buffer at pH 5.0 and filter paper stripes (Whatman1) with a weight of 50 mg were used. After 15 min preheating the enzyme solution was added and after 1 h of hydrolysis the reaction was stopped by heating in a thermoblock at 95 °C. The glucose conversion was determined by HPLC analyses using the device from Shimadzu and applying the same conditions as described in chapter 4.3.2 HPLC analysis (Sugar analysis after enzymatic hydrolysis). The measurements resulted in 252 FPU/ml for the enzyme solution.

For the determination of the residual free protein in the supernatant after enzymatic hydrolysis (see chapter 5.15) the same device and method to determine the total organic carbon was applied as described above.

4.2.9 Enzymatic hydrolysis

General procedure

All enzymatic hydrolyses were performed with a pulp load of 2 % (dry) using a sodium acetate buffer with pH 5 and a temperature of 50 °C. The reactions were ended by heating the respective samples at 90 °C for 10 – 12 min. However, the working volume, the enzyme load, and the sample taking time varied between the experimental scenarios. Additionally, additives were added to the reaction or the initial material was modified. The different EH scenarios are listed in Table 3.

Experiments with a work volume of 200 ml were performed in sealed Erlenmeyer flaks, which were shaked at ~ 1.35 rcf (Infors Multitron, Infors HT or lab shaker from Thermo Fischer Scientific with a heater from VWR). The experiments with a work volume of 10 ml were performed in 15 ml tubes, including three beads, which were mixed by a 360° rotation rotator device. The rotation was set to 23 rpm (Thermo Scientific Tube Revolver Rotator, Thermo Fisher Scientific) and the rotator was placed in a shaker for heating (Infors Multitron, Infors HT). For the sample taking in the EH scenarios 1 – 8, an Eppendorf pipette was used with cut pipette tips.

EH scenarios 2 – 8 were performed as double determinations and the EH scenarios 1 and 9 – 12 were performed as triplets. In graphs the results from double determinations are presented by marking the two individual values with symbols and the average value is shown

as a bar or line. Graphs of the experiments, which were performed in triplets, include the average values and the respective standard deviation as error bars.

Sugar analyses were performed by HPLC analyses (see chapter 4.3.2). For evaluation, the detected glucose amounts were corrected by the substrate and enzyme blank and shown as glucan conversion per gram of glucan [mg/g] in the SPS (determined by acidic hydrolysis). Monomeric glucose was transformed to glucan by the factor 0.9 (NREL, 2015). Potentially detected cellobiose was not included in the evaluations.

$$C = \frac{((A_{EH} - B_E - B_S) * f)}{A_{AH}}$$

C = Sugar conversion per gram glucan in the SPS [mg/g]

A_{EH} = Amount of monomeric sugar detected after enzymatic hydrolysis [mg]

B_E = Amount of monomeric sugar detected in the enzyme blank [mg]

B_S = Amount of monomeric sugar detected in the substrate blank [mg]

f = factor to convert monomeric to polymeric sugars

A_{AH} = Amount of polymeric sugar detected after acidic hydrolysis [mg]

Modifications

For the EH scenarios 3-5, 8, 11-12, which included the additives BSA (protease free, VWR) and Tween®20 (Sigma-Aldrich) the respective additives were first solved in buffer solution to receive a stock solution. The final concentration of BSA or Tween20 in the flasks is included in Table 3.

For the EH scenarios 7 and 8 the wet SPS material was intensively washed prior to enzymatic hydrolysis. For the washing the SPS was mixed with warm water (50 °C) and washed under continuous stirring for 30 min and filtered afterwards. This washing procedure was repeated two times, to reach three washing circles in total. The ratio between SPS and water was 1:80 based on dry SPS.

For the EH scenarios 10 and 12 extracted SPS material was used. For the extraction wet SPS material was mixed with Ace/H_2O and extracted under continuous stirring for 24 h. The solvent mixture was prepared in the ratio 7:3, considering the water content of the sample. The ratio between sample material and solvent was 1:14 based on dry SPS. After the extraction, the material was filtered using filter paper from Machery-Nagel (MN 640d, Blue ribbon, ashless) and washed three times with fresh deionized water for 2 min under stirring, to remove the acetone.

Table 3: Applied scenarios for the enzymatic hydrolysis experiments (EH scenarios).

EH scenario	Working volume	Pulp (dry) load	Enzyme load	Material	Additives	Enzyme addition	Sample taking time	Samples
1	200 ml	2 %	6 %	SPS	---	directly	2h, 4h, 6h, 8h, 24h, 48h, 72h	all
2	200 ml	2 %	8 mg/g* 12 FPU/g#	SPS	---	after 1h	2h, 4h, 6h, 8h, 24h, 48h	all
3	200 ml	2 %	8 mg/g* 12 FPU/g#	SPS	Tween20 (c=2.5 g/l)	after 1h	2h, 4h, 6h, 8h, 24h, 48h	all
4	200 ml	2 %	8 mg/g* 12 FPU/g#	SPS	BSA (c=50 mg/g dry pulp)	after 1h	2h, 4h, 6h, 8h, 24h, 48h	all
5	200 ml	2 %	8 mg/g* 12 FPU/g#	SPS	Tween20 (c=1.25 / 5.0 g/l)	after 1h	2h, 4h, 6h, 8h, 24h, 48h	oak, chestnut (db)
6	200 ml	2 %	8 mg/g* 12 FPU/g#	SPS	Ellagic acid (c=0.011 mg/ml)	after 1h	2h, 4h, 6h, 8h, 24h, 48h	Beech (db)
7	200 ml	2 %	8 mg/g* 12 FPU/g#	Intensively washed§ SPS	---	after 1h	2h, 4h, 6h, 8h, 24h, 48h	oak, chestnut (db)
8	200 ml	2 %	8 mg/g* 12 FPU/g#	Intensively washed§ SPS	Tween20 (c=2.5 g/l)	after 1h	2h, 4h, 6h, 8h, 24h, 48h	Chestnut (db)
9	10 ml	2 %	8 mg/g* 12 FPU/g#	SPS	---	directly	48h	beech, oak (db)
10	10 ml	2 %	8 mg/g* 12 FPU/g#	extracted° SPS	---	directly	48h	beech, oak (db)
11	10 ml	2 %	8 mg/g* 12 FPU/g#	SPS	Tween20 (c=2.5 g/l)	directly	48h	beech, oak (db)
12	10 ml	2 %	8 mg/g* 12 FPU/g#	extracted° SPS	Tween20 (c=2.5 g/l)	directly	48h	beech, oak (db)

* mg protein per gram of glucan
# FPU per gram of glucan
° SPS after Ace/H_2O extraction (chapter 4.2.3)
§ SPS after intensive washing with deionized water

4.2.10 Determination of particle size distribution

The SPSwp samples were first washed for 30 min by adding 4 l of 50 °C warm water to 50 g material, based on dry weight. The material was filtered under vacuum and transferred to a standardized disintegration mixer using 2 l of cold tab water. The mixer ran for 30,000 rotations. The disintegrated sample was transferred to a storing container using a 2 mm sieve to sort out particles > 2 mm.

In the following, the disintegrated sample was analyzed at the Anton Paar 1190 LD system using the liquid sample injection mode. As reconstruction mode Mie was chosen as well as a medium pump and stirrer speed. For the material characteristics, a refractive index of 1.6 was set, as well as an absorption coefficient of 0.1000 1/m for lignin. Further, the sample was dispersed prior to the measurement for 1 min and during the measurements, the ultrasound was turned on. Each sample was injected three times and each injection included 6 measurements in a row.

4.2.11 Determination of substrate accessibility

The accessibility of the substrate was investigated by solute exclusion method based on the principle introduced by Stone and Scallan (1967, 1968b). The practical application was leaned on the processing described by Grous et al. (1986) and Ishizawa et al. (2007), but was adapted to the laboratory circumstances. Individual solutions with solutes of different molecular sizes were prepared with known concentration. These solutions were mixed with a defined amount of wet SPS material, based on the dry content. Dependent on the molecule size the substances penetrated into the substrate pore structure. As a result, under consideration of the total water content of the sample the amount of water, which is not accessible for the molecules (inaccessible water), was determined by evaluating the change of substance concentration in the solution.

A molecule with a size bigger than wood pores was chosen to determine the maximum value for inaccessible water. By subtracting, the individual values of inaccessible water determined for the respective solutes, from the maximum value, the amount of water accessible to the molecules (accessible water) was calculated. The results were given in ml per g of dry material. For calculation, the following equation of Stone and Scallan (1968b) and Grethlein et al. (1984) were used:

Inaccessible water (Stone & Scallan, 1968b):

$$\delta = \frac{(\omega + q)}{p} * \left[1 - \frac{\omega}{\omega + q} * \frac{c_i}{c_f}\right]$$

Accessible water (Grethlein et al., 1984):

$$A_i = d_{560} - d_i$$

δ = inaccessible water in gram per gram of dry pulp

ω = weight of dextran solution added to the pulp [g]

q = weight of water in the pulp sample [g]

p = dry weight of the pulp sample [g]

c_i = initial concentration of the dextran solution [g/g]

c_f = concentration after the mixing with the pulp [g/g]

A_i = accessible water [g/g]

d_{560} = amount of inaccessible water [g/g] of the biggest dextran molecule with a size of 56 nm

d_i = amount of inaccessible water of another solute solution [g/g].

In Table 4 the used solute molecules, which comprised different dextrans and sugars are summarized. The table further shows their molecular mass and size. The molecular mass was information from the producer and the size values have been taken over from Stone and Scallan (1968b), who calculated these based on the Einstein-Stokes formula, which is based on the diffusion coefficients of the dextrans. The diffusion data for the substances was prior determined by other researchers (Granath & Kvist, 1967; Longsworth, 1952). The size values for D70 and fructose were taken over from Grous et al. (1986).

Table 4: Overview on the used solute molecules for substrate accessibility experiments.

Substances	**Molecular mass** [g/mol]	**Size** [nm]	**Producer**
D2000 (D5376) (Dextran from Leuconostoc mesenteroides)	~ 1,500,000 – 2,800,000	56	Merck (Order number: D5376-100G)
D500	~ 500,000	27	VWR (Order number: J63702.09)
D70 (pure)	~70,000	11	Carl Roth (Order number: 7616.1 & 9228.3)
D10 (for biochemistry)	~ 40,000	5.1	
Raffinose (99 %)	504.42	1.2	Alfa Aesar
Fructose (for biochemical and microbiological purposes)	180.16	0.8	Merck

For the procedure first, the dry content of the wet SPS samples was determined using the vacuum oven method (chapter 4.2.1). Next, 5 g of the SPS, based on dry content, was weighted into 50 ml plastic flasks and 15 g of 2 % dextran solution (weight based) were added. The flasks were mixed for 24 h on a GFL 3020 orbital shaker (amplitude 32 mm) set to 150 rpm. Each sample was prepared as triple. Before measuring the sugar concentration by refractor index measurements, the samples were left for 15 – 20 min to let the solid material settle. The supernatant was taken with a 5 ml syringe and filtered through 0.2 µm PES filters (WICOM). The sugar concentration was then analyzed using a modified UltiMate 3000 HPLC system (see chapter 4.3.2).

4.2.12 Bark proportion determination

The bark proportion for the samples was retrospectively estimated using a mathematical formula based on the research of Altherr et al. (1974), which uses the average diameter of a tree section (including bark) to calculate the bark proportion of standing trees. The available harvesting lists contained the diameter of the different logs after subtraction of the bark dimension. Therefore, the proportion of the bark was added to the listed diameter following the guidance values of the “Rahmenvereinbarung Rohholzhandel (RVR) (= Framework agreement

about raw wood trade)“ (DFWR & DHWR, 2015) to get the correct initial values for the formula of Altherr et al. (1974) cited in (Rössler, 2008).

$$Ri = 20 * \left[\left(\frac{A}{DM}\right) + B + C * DM\right] * \left[1 - 0.05 * \left(\left(\frac{A}{DM}\right) + B + C * DM\right)\right]$$

$\mathrm{Ri} = \text{percentage of bark [\%]}$

$\mathrm{DM} = \text{diameter under bark [cm]}$

$\mathrm{A, B \text{ and } C} = \text{individual, mathematical factors for each tree species (see Table 5)}$

As the literature contains no individual factors for the oak species English oak (*Quercus robur*) the same values as for Sessile oak (*Quercus petraea)* on clay soils are used. For chestnut, no individual factors are available, too. Thus, the values for beech are used as a best approximation taking into account the probable age and bark developing stage of the tree.

Table 5: Mathematical factors for different tree species for the formula of Altherr et al. (1974) to determine the bark content of standing trees.

Species	A	B	C
Beech *(Fagus sylvatica)*	2.61029	0.28522	0
Sessile oak *(Quercus petraea) (on clay soils)*	9.88855	0.56734	0
Ash *(Fraxinus excelsior)*	-7.97623	1.40182	-0.01011

4.3 Instrumental analytics

This chapter contains the detailed information about the conditions of the different instrumental analytics applied in combination with the methods of material characterization and analysis.

4.3.1 FTIR analysis

FTIR analyses were performed on the device iS 50 FTIR Nicolet from Thermo Fisher Scientific. The samples were mixed with potassium bromide (KBr) and pressed to tablets. The samples were measured between 4000 – 400 $cm^{-1}$and the background with a pure KBr tablet.

Spectra evaluation was performed within the software Opus (version 7.2) from Brucker Corporation. All spectra were base line corrected. For statistical evaluation, a Min-Max normalization was performed and a total of 12 data points for each sample were included. For principle component analysis (PCA), the software Unscrambler X (version 10.5.46655.794) was used. Further, the data was manipulated by first derivatization (Savitzky-Gelay) and analyzed within the range of 1800 - 400 cm^{-1}.

4.3.2 HPLC analysis

Sugar analysis after acidic hydrolysis

An aliquot of the filtered hydrolysate was neutralized with $CaCO_3$ to a pH of 5 - 6. The supernatant was filtered through a syringe filter (PES, 0.2 µm, WICOM). This sample was analyzed for sugars at a Knauer Smartline HPLC system, including a pump 100, a RI detector 2300 and an extra column compartment from Hitachi (L-7300). The system was equipped with an Aminex HPX87-P column for the analysis of monosaccharides (Bio-Rad Laboratories). The system ran with ultrapure water, a flow of 0.6 ml/min and an oven temperature of 80 °C. The method for the HPLC analyses was based on the instructions in NREL (2008) about the determination of structural carbohydrates and lignin. The data was evaluated with ClarityChrom software from Knauer (version 8.1.0.87). Each sample was injected twice.

Analysis of sugar degradation products

The LPS samples were diluted (1 : 6) with ultrapure water and filtered through a 0.2 µm PES filter from WICOM prior to analysis. Analyses ran at an UltiMate 3000 HPLC system including an UltiMate 3000 Pump (LPG-3400A), an UltiMate 3000 Autosampler (WPS-3000 SL Analytical), an UltiMate 3000 RS Column Compartment (TCC-3000 RS) as well as an UltiMate 3000 RS Variable Wavelength Detector (VWD-3400RS) and an RI detector (RefractoMax 520). The system was from Thermo Fisher Scientific.

For the measurements, 10 µl of the sample were injected minimum two times and analyzed on an Aminex HPX-87H analytical column (300 mm x 7.8 mm) (Bio-Rad Laboratories). The system ran with 5 mM H_2SO_4, at 60 °C and with a flow of 0.6 ml/min. Detection was done by an UV detector at 210 and 254 nm. The procedure was based on the instructions of the NREL about the determination of sugars, byproducts, and degradation products in liquid fraction process samples (NREL, 2006).

Measurements for the solute exclusion method

The UltiMate 3000 system, described above was used, but was slightly modified. For the detection of the RI signal of the dextran or sugar solutions, the analytical column was bridged and the system ran with ultrapure water and a flow of 0.4 ml/min. Each sample was at least injected 5 times with an injection volume of 5 µl and one run took 3 min. The absorbance value detected by the RI detector was then used to evaluate the change in concentration based on an individual calibration curve for each solute molecule (four point calibration).

Sugar analysis after enzymatic hydrolysis

In all scenarios 1 – 12 (see Table 3) the sugar contents were analyzed with an analytical Aminex HPX-87P column (Bio-Rad Laboratories) for monosaccharides and the system ran with ultrapure water at 80 or 85 °C, applying a flow of 0.6 ml/min. Analytical conditions were based on the instructions of (NREL, 2008). However, the HPLC system used for analysis varied between the experiments: For the analysis of scenario 1 and 5 to 12 a Shimazu HPLC system including the pump LC-40D xR, a column oven (CTO-40S) and a autosampler (SIL-40XR) was used. The detection was performed with a RI detector (RID-20A) and for evaluation the LabSolutions software (version 5.99) was applied.

For the other scenarios 2 – 4 the HPLC system Thermo Vanquish HPLC, including an ERC-RI detector (RefractoMax 520) (Thermo Fischer Scientific) was used. The system control and evaluation was done with the Chromeleon software (version 7.2).

4.3.3 Analysis of reducible monomeric sugars in the LPS

The analyses were performed for the LPS samples after SPE using the extracted phase (see chapter 4.2.3) at a modified LC 3000 Amino Acid Analyser (Laborservice Onken). The separation method is based on Uremovic et al. (1994). The sugars are separated on an analytical ion exchange column by different buffer solutions via borate complexes. Afterwards the reducing sugars in the sample react with a reagent, which contains copper and biquinolate. The reducible sugars oxidized the copper, which forms color complexes with biquinolate. The formation of the color complex correlated with the sugar concentration and is detected by a photometer between 560 – 570 nm.

4.3.4 UV measurements for ASR determination

UV device (model: UV-2450) from Shimadzu. For the UV measurements, 10 mm cuvettes were used with deionized water as background. For each sample two dilutions were prepared and measured three times, scanning from 400 – 190 nm. The absorption value was taken at 205 nm and an absorptivity constant of 110 $lg^{-1}cm^{-1}$ was applied for calculation, following the equation:

$$ASR[\%] = \frac{Ab * Vol * dilution}{\varepsilon * weight * way}$$

Ab = absorbance $[lg^{-1}cm^{-1}]$

Vol = volume of the hydrolysate [l]

ε = Absorptivity constant $[lg^{-1}cm^{-1}]$

weight = dry weight of the sample [g]

way = thickness of the cuvette [cm]

4.3.5 (Pyrolysis) GC/MS analysis

All GC/MS analyses were performed at an Agilent Technologies 7890A GC System connected to an Agilent Technologies 5975C VL MDS with Triple-Axis Detector. The system used helium as carrier gas. For the measurement and evaluation, the MSD ChemStation E.02.02.1431 software from Agilent was used. For the first identification of the MS spectra, the NIST Mass Spectral Search Program for the NIST/EPA/NIH EI and NIST Tandem Mass Spectral Library (version 2.4) was applied.

For liquid injection mode, the GC/MS device was equipped with a BPX5 column (30 m x 0.25 mm x 0.25 µm) from Trajan Scientific and Medical as well as an auto sampler from Agilent Technologies (G4513A ALS).

For pyrolysis mode, the device was equipped with a Selective Sampler SS - 1010E and a Double - Shot Pyrolyzer PY-2020iD from Frontier Lab (Koriyama, Japan). For pyrolysis control, the software PY-2020iD Control (Version 3.02) purchased from the same manufacturer was used. The analytical column VF-17 ms (30 m x 0.25 mm x 0.25 µm) was from Agilent Technologies (J & W Columns).

Method A

The sample was solved in N,N-Dimethylformamide (DMF) anhydrous 99.8 %. For derivatization N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA 99 %, TMCS 1%) was used, suited for GC derivatization. Solvent and silylation reagent were purchased from Sigma-Aldrich. The sample was heated for 1 h at 80 °C to complete the reaction.

The silylated sample was measured at the GC/MS device using liquid injection mode with an injection volume of 1 µl (split 30:1) and an injection temperature of 320 °C. Over the analysis time, the temperature was increased from 100 °C to 320 °C with a heating rate of 10 °C/min. The starting temperature was hold for 1 min and the end temperature for 10 min.

Method B

Sample preparation was equal to Method A, but the GC/MS measurement conditions were adapted as followed: the injection temperature was 340 °C (split 30:1). The starting temperature of 100 °C was hold for 1 min and ramped with 10 °C/min till 240 °C, hold for 1 min and ramped further with 20 °C/min to 350 °C, which was hold for 20 min.

Method C

The sample preparation is similar to Method A, except for the solvent, where dichloromethane (DCM) (Supelco for gas chromatography, Merck) was used. The measurement conditions were also similar to Method A, but the starting temperature was reduced to 50 °C.

Method D – Pyrolysis GC/MS

100 – 200 µg of extract was weighted in pots and were injected in the pyrolyzer oven at 450 °C for 0.2 minutes under helium (split 40:1). The starting temperature of 50 °C was hold for 1 min and ramped with 10 °C/min up to 300 °C. The end temperature was hold for 4 min.

Method E – Pyrolysis GC/MS

Around 100 – 200 µg of extract was weighted in pots and 7 µl of derivatization reagent was added for on-line silylation. Measurement conditions were similar to Method D.

4.3.6 GC analysis

The GC analyses are performed on a GC-2010 Shimazu system running with helium and is equipped with a BP5 (30 m or 15 m x 0.25 mm x 0.25 µm) analytical column. For detection, a FID detector running with hydrogen is applied.

Method F

The injection temperature is set to 300 °C and the temperature of the FID detector to 310 °C. During the measurement, the column runs isocratic at 115 °C.

Method G

The injection temperature is set to 300 °C, as well as the FID temperature. During the run, the temperature ranges from 140 – 300 °C.

4.3.7 Analysis of mineral content

Energy dispersive X-ray analysis (EDXA)

Element identification of ashes (wood and SPS) was performed by energy dispersive X-ray spectroscopy (EDX) on an EVO 40 system from Zeiss. For the element identification, the system was additionally equipped with a Bruker XFlash Detector 5010 (Bruker Corporation, Billerica) with a resolution of 129 eV. The data was evaluated with the software ESPRIT 1.9 from Bruker Corporation and SmartSEM (Vo5.04.03.00) from Zeiss.

ICP analyses

The elemental composition analysis of wood powder, of the SPS and LPS was performed by the TUM Centre for Building Materials.

For sample preparation, the solid samples were dried and milled prior to the following digestion reaction: 200 mg dry sample were used to perform a microwave digestion using 10 ml nitric acid (65 %) and 1 ml hydrogen peroxide solution (30 %). The solution was added to the sample material and after 15 min, the reaction flask was closed. The flask was heated for 15 min to 210 °C in a microwave oven (model: Mars6) from CEM and the end temperature was hold for 20 min. After a cooling period of 15 min the samples were diluted with water to reach an end volume of 250 ml.

For liquid samples, an aliquot of 5 ml was taken and diluted with 1 ml nitric acid (65 %) to an end volume of 50 ml.

With an ICP-OES device from Perkin Elmer (Avio500) the elements Na, K, Ca, Mg, Fe, Al, S, P, Ba, Sr, Mn, Ti, Cr, V and Zn were determined, whereas an ICP-MS device (Perkin Elmer, Nexion 300D) was implied to analyze the elements As, Be, Cd, Co, Cu, Hg, Mo, Ni, Pb, Sb, Se, Sn, Te, TI and W. For the determination of the chloride content, a chloride cuvette test for photometric measurements was used. The photometer device (model: DR6000) and the cuvette test (type: LCK311) were from Hach-Lange.

4.3.8 Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE)

Sample preparation

After enzymatic hydrolysis the tubes were shortly centrifuged to separate the supernatant from the solid residue. An aliquot of the supernatant was concentrated by the factor of 10 using a ViVASPIN6 Centrifugal concentrator (Sigma-Aldrich). Centrifugation was done at Megafuge 40R Centrifuge (Thermo-Scientific) at 4000 rcf and 15 °C. These concentrated samples were properly mixed with five-fold Laemmli buffer (4 parts sample to 1 part buffer) and boiled at 95 °C for 5 min using a Thermo shaker TS-100C from Krisker Biotech GmbH & Co. KG. After boiling, the samples were directly transferred on ice and stored at -20 °C until analysis.

The Laemmli buffer was prepared as followed: 312 mM tris-hydrochlorid (tris-HCl, pH 6.8) was mixed with 50 % glycerol ,10 %SDS (w/v) and 0.02 % bromophenol blue. After mixing with 15 % 2- mercaptoethanol the buffer was stored in a freezer at -20 °C. The tris-HCl (Tris Pufferan® ≥ 99,9 %, p.a.) was purchased from Carl-Roth as well as the SDS ($C_{12}H_{25}NaO_4S$, ultra pure ≥ 99% for electrophoresis, biochemistry and molecular biology) as well as the

glycerol (anhydrous, ≥ 99.5 % for analysis, Rotipuran®, Carl-Roth). The bromophenol blue - sodium salt (for laboratory use) was from SERVA and the 2-mercaptoethanol was purchased from Sigma-Aldrich (≥ 99 %)

Preparation of the separation gel

For the separation gel, a cross-linking between the acrylamide / bis solutionof 10 % was chosen. 3.8 ml of double deionized water was mixed with 3.4 ml of acrylamide / bis solution (37.5 : 1, 30 % w/v, 2.6 % C, for laboratory use, SERVA). Additionally, 2.6 ml of 1.5 M tris hydrochloride (tris – HCl, pH 8.8) and 0.1 ml of 10 % (w/v) SDS solution was added. To start the curing 0.1 ml of 10 % (w/v) ammonium peroxidsulfate (≥ 98 %, p.a., Carl-Roth) and 0.01 ml TEMED (≥ 99 % for electrophoresis, Carl-Roth) were added. The prepared gel amount was enough for two 1 mm gel compartments with 10 wells.

Preparation of the stacking gel

For the stacking gel 2.975 ml of double deionized water were mixed with1.25 ml of 0.5 M Tris-HCl (pH 6.8) and 0.05 ml of 10 % (w/v) SDS solution as well as with 0.67 ml acrylamide / bis solution. For curing 0.05 ml of 10 % (w/v) ammonium persulfate and 0.005 ml of TEMED were added. The prepared gel amount was enough for two 1 mm gel compartments with 10 wells.

Loading and running of the gels

After curing, the gels were transferred to the electrophoresis chamber. For the running buffer (10 x concentrated) 30 g of TRIS Pufferan® ($C_4H_{11}NO_3$, ≥ 99.9 %, for analysis, Carl-Roth) 144 g of glycine (≥ 99 %, for biochemistry, Carl-Roth) and 10 g of SDS were solved and brought to a volume of 1000 ml with double deionized water. Before purring the buffer into the chamber it was diluted 10 times. For scaling, the protein marker BlueStar Prestained Protein Marker from Nippon Genetics EUROPE (Lot 13 3001 101200) was used. For each run 15 µl of the sample were added into the wells and 7.5 µl of the protein marker. The gels ran in a Mini-Protean® Tretra System from Biorad and the running conditions were set to 100 V and 0.5 A.

4.3.9 Coomassie® blue staining of SDS gels and evaluation

The SDS gels were stained for 1 h with a staining solution containing 0.1 % Coomassie® Blue, 40 % ethanol and 10 % acetic acid. The used EtOH was GC grade (≥ 99.8 %, Merck) and the acetic acid was purchased from Carl-Roth (Rotipuran ®·for analysis, 100 %). The Coomassie® Brilliant Blue R250 was purchased from Serva. After staining the gels were transferred to a destaining solution, which contained 10 % EtOH and 7.5 % acetic acid. The gels were destained till the background was reduced and the protein bands best visible. Destaining was partly speeded by short microwave heating. The protein separation was visualized by recording images using the gel doc device Fusion Solos Vilber Lourmat and the software EvolutionCapt Solo (version 6S17.04a).

5. Results and Discussion

This chapter presents the results and a first discussion of the individual analyses. The chapter is subdivided into three parts, working on different topics in relation to the research areas and individual research questions (Rq). An overview on the frame of this thesis and the included analyses structured by the research questions is included in the appendix (Figure 54, page 196) as a summary of Figure 5, Figure 22 und Figure 38.

Part 1: Species usability, component distribution and influence of bark

This first part of the result and discussion chapter focuses on research area 1 and 2, including the individual research questions (Rq), except 2.3. The results mainly comprise analyses to characterize the different material streams regarding species-specific differences and the respective influence of bark. Further, the suitability of the different materials to produce glucose as the main platform substance was evaluated. The following Figure 5 includes the first two Rq and an overview on the performed analyses in relation to the different materials.

1. The first research area worked on the usability, including suitability and potentials of the different tree species as raw material for biorefinery processes, which target the utilization of the individual wood components.
1.4. How to evaluate the suitability of the different tree species for the production of the main platform glucose by enzymatic hydrolysis?
1.5. Which species-specific patterns are detectable in the liquid and solid process stream?
1.6. How does the pretreatment process distribute the different components within the process streams?

2. **The second research area focused on the influence of bark on the processing of the material during pretreatment and enzymatic hydrolysis in terms of profitability and additional potentials**.
2.1. How does the presence of bark influence the suitability of the tree species for the production of the main platform glucose by enzymatic hydrolysis?
2.2. How does the presence of bark influence the composition of the different process streams?
2.3. Which effect does the presence of bark have on the factors influencing enzymatic hydrolysis?

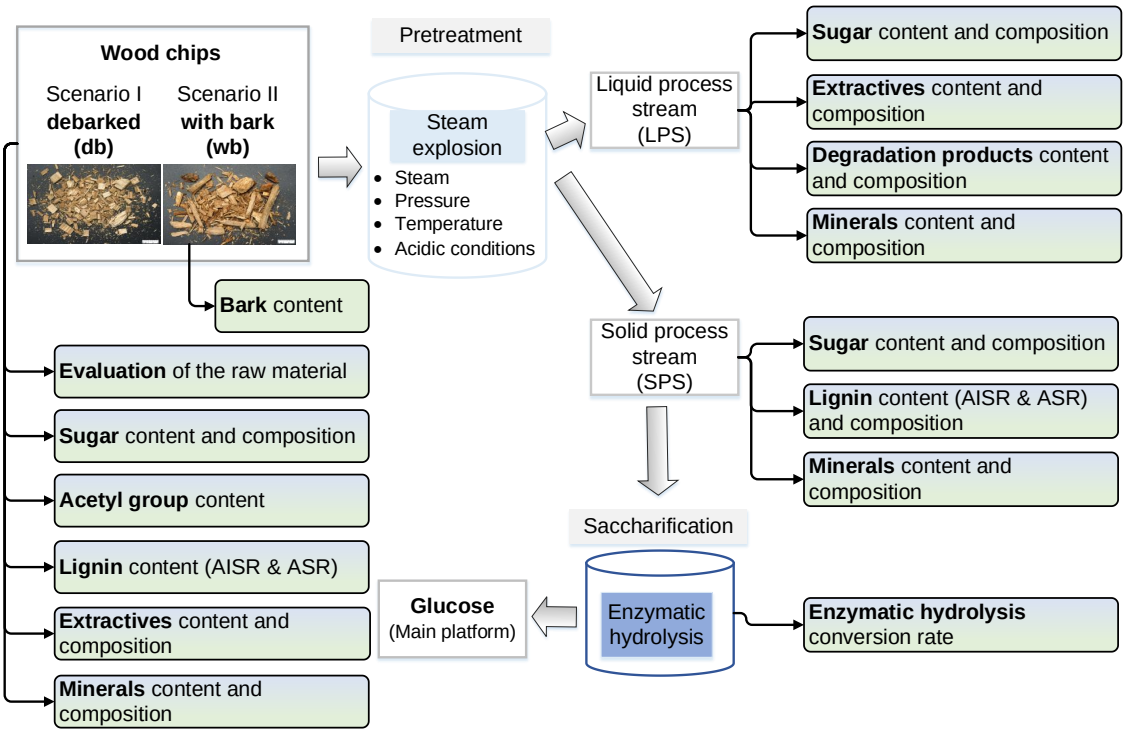


Figure 5: Overview on included analyses to work on part 1: Species usability, component distribution and influence of bark.

5.1 Evaluation of the raw material and recalculation of bark content

The evaluation of the harvesting lists showed, that several trees per species were harvested to reach the needed material volume for further processing. This was advantageous as the mixture of different tree species leveled potential effects caused by the natural variability between tree individuals. However, variability effects of the growing site were not leveled as the trees were harvested in the same region.

The harvesting lists showed an average diameter of 44 cm for the processed logs of beech and oak. For ash, the average diameter was 33 cm and for chestnut 21 cm. Considering, the secondary thickness growth in correlation with the trees **age** it was assumed that especially the chestnut trees were younger than the respective ash, oak or beech trees. Differences in age can have an impact on different properties of wood and bark like the extract content or the polymerization degree of tannins. Further, it influences the proportion of juvenile wood or the ratio between wood and bark proportion (Klumpers et al., 1994; Mai & Zhang, 2023).

This assumption was supported by the appearance of the bark particles within the samples (Figure 6). For beech, a thin and smooth bark structure was observed and for ash and oak, the pieces indicated a thicker and rougher bark structure. The observations for beech were typical for this tree species, as beech keeps its initial periderm. Contrary, oak and ash form a secondary periderm and thus the bark becomes thicker and shows more texture with tree aging (Godet, 2011; Wagenführ, 1984). The recorded diameters in combination with the observed bark characteristics indicated a mature bark structure for beech, oak and ash. For chestnut, the included bark pieces showed a thin and smoother surface, similar to beech, despite the fact, that chestnut forms a secondary periderm similar to oak or ash. The observed conditions would be typical for young chestnut trees, as the bark is thin and smooth during youth, but develops a thicker and rougher netlike structure with aging comparable to oak and ash (Fengel & Wegener, 2003; LWF, 2018; Moeller, 1882). Therefore, the observed bark characteristics for chestnut supported the assumed age difference between the harvested trees.

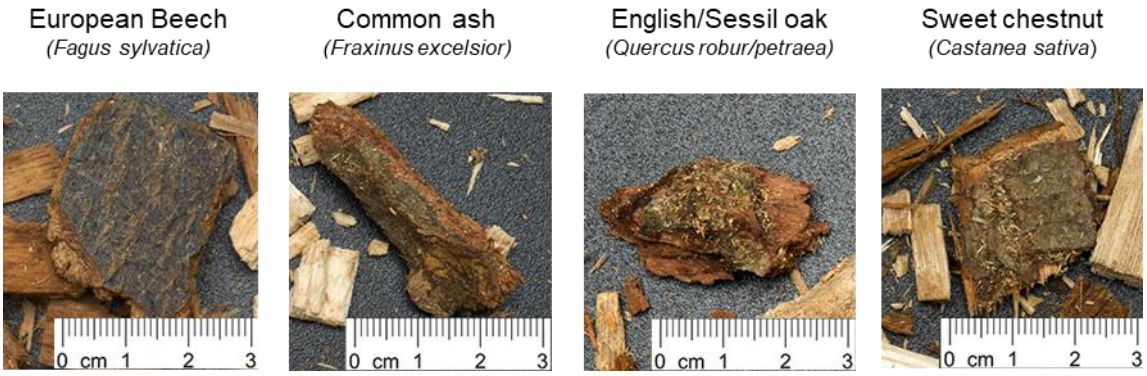


Figure 6: Examples of bark pieces found in the wood chip batches with bark

To enable a better evaluation of the influence of bark on the different processes, the **bark proportion** of the logs was recalculated using the formula of Altherr et al. (1974) cited in Rössler (2008). For this recalculation the trees diameter without bark originated from the harvesting lists and the official values for bark dimensions (see chapter 4.2.12) were used. According to the tree species, the respective calculation factors were applied for beech, oak and ash, but there were no individual values for chestnut available. The same calculation factor as for beech was chosen for chestnut. This was because, of the supported assumption of the young age of the harvested chestnut stems and the resulting smooth and thin bark structure, which is comparable to the structure of beech bark. Additionally, the given factors were solely

described for Sessile oak. However, the Sessile oak and English oak are commonly not separated during the formation of wood assortments and thus the values were used for the present material as well. Table 6 presents the average stem diameters of the harvested trees without bark and the calculated bark proportion.

Table 6: Average diameter (DM) without bark and the calculated bark proportion of the stem sections.

Tree species	**Ø DM db**	**Bark proportion**
	[cm]	[%]
Beech	44.0	6.7
Ash	32.8	15.5
Oak	44.1	15.0
Chestnut	20.7	8.1

db = debarked

Ash and oak, the species with the naturally thickest bark structure in adult state showed the highest bark content with ~ 15 %. In contrast, beech as a species, which keeps its initial periderm, showed a very low bark content of 6.7 %. The value for chestnut was slightly higher than for beech with 8.1 %. This was because the diameters of the chestnut stems were thinner than of beech and thus the proportion of bark compared to the wood volume was bigger for chestnut, than for beech. This resulted in a higher bark proportion. In literature, the bark proportions of beech *(Fagus sylvatica)* were given with 6 - 9 % and for ash with 9 - 14 %. 12 - 14 % for oak, which agreed with the here determined values. Typical values for softwoods were in the range of ash and oak with for example 10 % for spruce or 12 – 14 % for pine (Wagenführ, 1984).

For following interpretation of the chemical analyses of the different samples and especially for the comparison between scenario I and II, (db and wb), it had to be considered that the stems were technically debarked prior to chipping, by a drum chipper. For scenario I (db) this meant, that the bark content was reduced to the technical possible minimum. The batch with bark (scenario II) in contrast contained the natural proportion of bark, which simulated the case of a skipped debarking process. The bark content thus depended mainly on the age of the tree, the species and the diameter. The use of material with bark, thus represented a potential economical favorable case for commercial applications. This was based on the assumption, that the costs for the acquisition and maintenance of the debarking unit as well as the separate downstream processing of the bark proportions could be omitted.

5.2 Sugars and lignin in wood

The sugar content and composition in wood was determined by acidic hydrolysis of water extracted wood meal. Simultaneously the acid insoluble lignin and the acid soluble lignin were determined. However, it has to be mentioned that for processed materials like the SPS the term lignin is misleading, as the acid insoluble and acid soluble proportion probably contain not solely lignin, but reaction products from extractives or sugar degradation. Further, the soluble fractions could contain other soluble substances with similar UV activity like aromatics around 205 nm. To consider these circumstances, the acid insoluble and acid soluble proportions analyzed in this thesis were uniform named “residues”. Therefore in the presented Figure 7 and Table 7 as well as in following similar figures and tables, the lignin fractions are named acidic insoluble residue (AISR) and acid soluble residue (ASR).

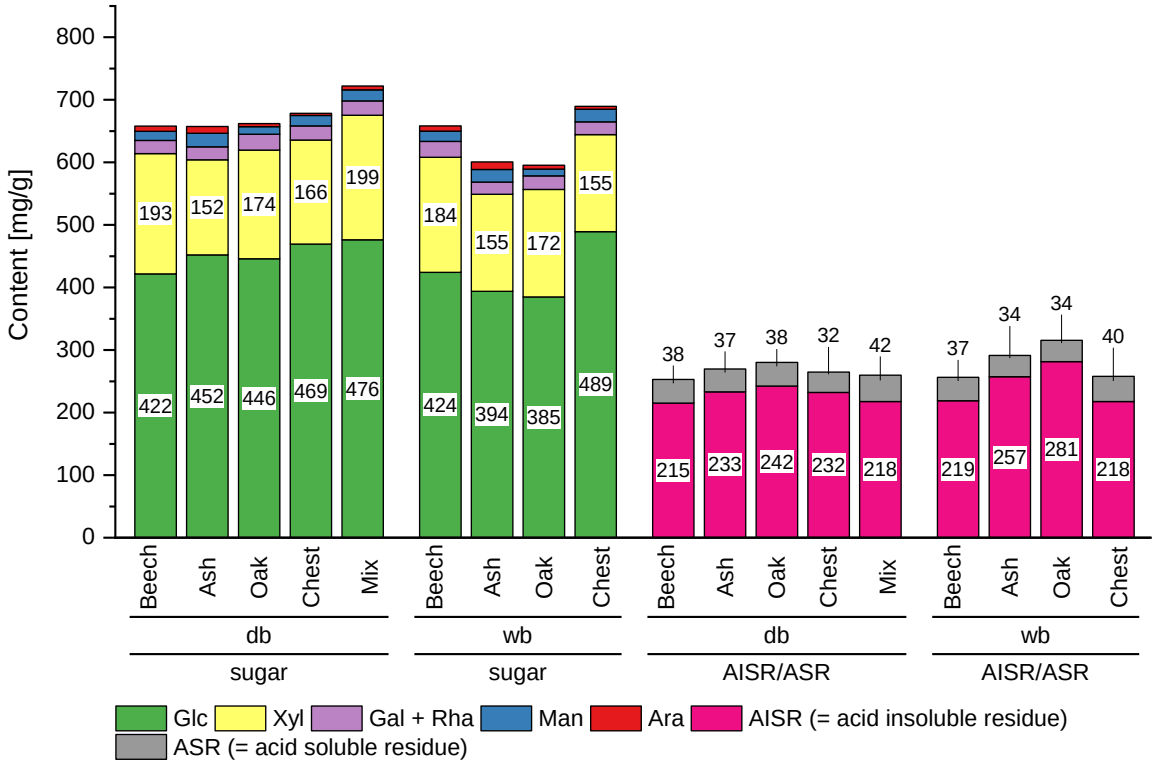


Figure 7: Results of acid hydrolysis of hot water extracted wood.

Contrary to most guidelines like NREL (2008) or TAPPI (1988) a hot water extraction was chosen instead of an extraction with organic solvents, to prepare the sample for the subsequent acid hydrolysis. This adaptation was made following the process conditions during the pretreatment, as the SE process does not apply organic solvents, but works with an acidic aqueous medium. There was no pH control during the hot water extraction, because an acidic hot water extract probably would pre-hydrolyze the sample and thus distort the results of the acidic hydrolysis. Additionally, accompanying tests compared the suitability of different extractions as preparation for subsequent acid hydrolysis of hardwoods. These tests showed that a prior hot water extraction led to similar or partly higher sugar recoveries compared to scenarios with no extraction or an extraction with ethanol or cyclohexane/ethanol (Lautenschläger et al., 2022). Moreover, no sugar correction factors were included in the calculations, because it was determined that the degradation rate of monomeric sugar

references were negligible. This was also confirmed by the RISE Biorefinery Proficiency Test 2022.

Table 7: Results of acid hydrolysis of hot water extracted wood powder.

Sample		AISR	ASR	Sum	Man	Ara	Gal + Rha	Xyl	Glc	Sum	% Recovery
						[mg/g]					[%]
Beech	db	215	36	251	15	8	21	193	422	658	91
Ash	db	233	37	270	22	11	21	152	452	657	93
Oak	db	242	45	287	12	5	25	174	446	662	95
Chestnut	db	232	32	265	17	4	23	166	469	679	94
Mix	db	218	42	260	18	6	23	199	476	722	98
Beech	wb	219	38	256	16	9	25	184	424	658	91
Ash	wb	257	34	291	20	12	20	155	394	601	89
Oak	wb	281	34	316	11	6	22	172	385	596	91
Chestnut	wb	218	40	258	20	5	20	155	489	690	95

ASR = acid soluble residue; AISR = acid insoluble residue; db = debarked; wb = with bark

The **total sugar** content for **debarked samples** was found to be highest for the mixed sample with ~ 72 % and for ash db and beech db as well as oak db and chestnut db the values were between 66 - 68 %. The mixed sample contained 60 % beech and 20 % ash and oak respectively.

Regarding the **glucan content,** a relatively low value was determined for beech db with 42.2 %. This did fit relatively with findings in literature, which showed cellulose values of 43.3 % or 49.1 % for *F. sylvatica*. The discrepancies between the reported values, pointed out the variations, which can occur due to different preparation, hydrolysis and calculation methods. Examples could be the usage of different acids or correction factors as well as the presentation of the values as monosaccharides or anhydrous.

Additionally, by comparing the cellulose values from literature with the here determined glucan values it had to be considered, that the glucan values comprise the glucose from cellulose and hemicelluloses like glucomannans. Glucomannans were reported with a molar ratio of 1 - 2 and 1. This means that for example for beech with a mannan content of 1.5 % (= 1.7 % mannose) approximately up to 1.7 - 3.4 % of the determined glucose (= 1.5 - 3.1 % glucan) was originated from hemicelluloses and not from cellulose. This has to be considered similar for the comparison of the reported cellulose values for *C. sativa* with 47.3 % and 37.9 % for *F. excelsior* as well as 41.1 % for *Q. robur*. Overall the value range in literature fitted quite well with the here determined values. (Fengel & Wegener, 2003).

For the **total sugar** content of **samples with bark**, clearly chestnut wb showed the highest values with ~69 %. In contrast, for oak wb and ash wb the lowest values were found with ~ 60 %. Interestingly, by comparing the individual sugar proportion of ash and oak the main discrepancy could be detected for the glucan. The proportion of glucan in the samples with bark is ~ 39 % smaller compared to the debarked samples. This is probably because of the high bark content of ash and oak (≥ 15%). In contrast, for beech and chestnut there was no strong indication of a reduced sugar or glucan content if bark was present, which correlates with the lower bark content of ~ 6 - 8 %.

An increased bark proportion would led to a smaller ratio of pure wood in the sample volume, used for the experiments. Additionally, the amount of cellulose and hemicelluloses in bark were found to be half of that in wood (Dietrichs et al., 1978). Dietrichs et al. (1978) found just a small difference in the kind of sugars present in bark or the wood xylem, but detected lower glucose contents of 29.7 % and 32.3 % for bark of beech and oak *(Quercus robur),* which is less compared to the glucose values of pure wood. However, it was found that the proportion of dissolvable sugars by different solvents like EtOH, H_2O or NaOH was higher in bark (Dietrichs et al., 1978).

However, in the presented analyses the amount of **hemicelluloses** seemed not to vary as much between debarked samples and samples with bark. For instance the xylan content of debarked samples was found to be 15 - 20 % and for samples with bark 16 – 18 %. Clearly, beech db and wb contained the highest xylan proportions within the single-origin samples. In literature, xylan values for wood of *F. sylvatica* and *F. excelsior* were found to be 27.8 % and 28.3 %. These values are slightly higher than the here detected xylan values, which is probably due to natural variations and varying analytical methods. For bark of *F. sylvatica* and *Q. robur* xylose values were reported with 20.1 % and 16.4 % (Fengel & Wegener, 2003), but direct comparison with the here presented values is not possible, because these are for bark samples and not for wood bark mixtures.

Regarding the **acid insoluble residue (AISR)** the values of the debarked samples were in the same range, but beech and the mixed sample showed the lowest proportions. The value for the mixed sample was probably due to the high beech content in the mixture (60 %). Interestingly the quantity of AISR determined for the samples including bark correlated positively with the determined bark contents. Highest AISR values were detected for ash and oak wb with ~26 % and ~ 28 %. Whereas for beech and chestnut with bark the AISR values were just slightly increased. The determined proportions for the AISR also agreed with the reported values for the lignin content in hardwoods with 17 – 25 %. However, increased values potentially could be related to non-extractable aromatic compounds (polyphenols), which were more frequent in samples with bark. In contrast, the values for **acid soluble residue (ASR)** seem not to be influenced by the presence of bark and were on the range of 3 – 4 %. In literature, a lignin content of 23.8 % for *F. sylvatica* was reported as well as 25.6 %, 29.6 % and 31.8 % for *F. excelsior*, *Q.robur* and *C. sativa*, which is in line with the here detected values (Fengel & Wegener, 2003).

Overall, there are slight differences detectable between the sugar content and composition of the different tree species. The effect of bark varies between the species and there is some indication that the effect of bark correlates with the bark proportion. Further, the high glucan proportions found for chestnut db and wb, indicate a good theoretical suitability in respect to the potential yield. These first findings relate to the research questions 1.1 and 1.2 as well as to research question 2.1.

5.3 Acetyl group content of wood

The determination of the acetyl group content is especially interesting for hardwoods as the content is higher than in softwoods (Fengel & Wegener, 2003).This functional group, mainly located at the hemicelluloses within wood, can be easily cleaved and form acetic acid, which supports autohydrolysis by cleaving glycosidic linkages. This reaction is also known for the SE process (Alvira et al., 2010; Berg & Guzmán, 2023; Cotana et al., 2015).

Table 8 presents the results of the quantification of the acetyl group content. Beech db and wb showed the highest acetyl group content with 4 % and oak db and wb showed the lowest acetyl group content with 2 - 3 %. This possibly indicated different hydrolysis potentials between the species. Interestingly, the presence of bark was not found to have a strong influence on the acetyl group content, which can be explained by the high proportion of wood in a sample. The determination of the acetyl group content contributes to research question 1.1.

Table 8: Mass fraction of acetyl groups in non-extracted wood

Sample db	**Acetyl groups [%]**	**Sample wb**	**Acetyl groups [%]**
Beech	4.27	Beech	4.39
Ash	3.27	Ash	3.24
Oak	2.81	Oak	2.44
Chestnut	3.20*	Chestnut	3.45
Mix	3.80		
db = debarked	wb = with bark	* relative deviation is > 5%	

5.4 Sugars and lignin in the solid process stream (SPS)

For the evaluation of the sugar content and composition as well as the lignin content, the SPS was directly hydrolyzed with sulfuric acid and analyzed at HPLC. Regarding the **sugar profile** (see Figure 8 and Table 9), glucose (glc) was the dominant sugar type. Except to some small proportion of xylose (xyl), other hemicellulose sugars were not detected in the range of calibration (0.02 – 0.2 mg/ml for mannose, arabinose and galactose). This was expected as hemicelluloses, especially xylans are more easily degraded than cellulose and the steam explosion process is known to solubilize hemicelluloses (Fengel & Wegener, 2003; Shafiei et al., 2015).

The sugar analyses showed the lowest **glucan values** for oak db and wb with 58.2 % and 47.6 %, whereas chestnut db had the highest glucan content with 67.0 %. The highest **xylan content** was found in beech db and wb with 3.3 % and 3.1 %, which was parallel to the wood samples, where beech also showed the highest xylan content compared to the other species. The detected xylan content could be partly a residual proportion, which was not solubilized during SE. This could be related to the process intensity, as the amount of residual hemicelluloses varies with different process conditions (Negro et al., 2003).

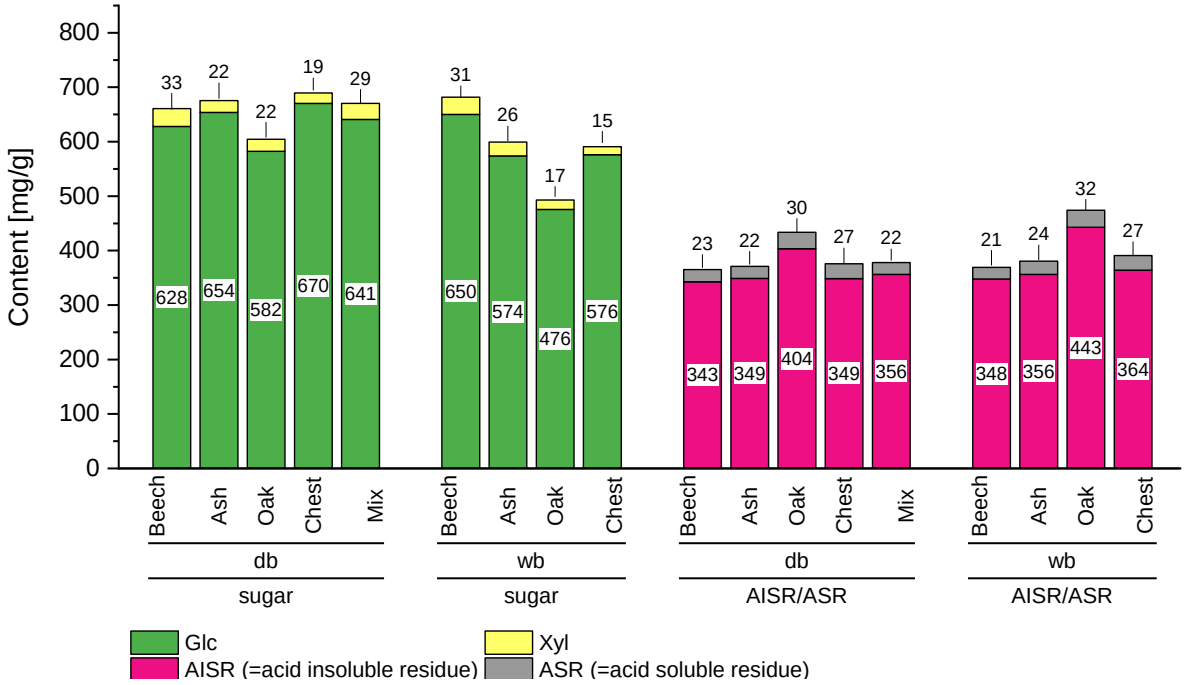


Figure 8: Results of acid hydrolysis of the SPS.

Cotana et al. (2015) analyzed three different oak species after SE using different severity factors. The glucan content was 41 - 45 % with increasing severity for *Q. ilex*, but in contrast to that, low-medium severity yielded the best results with 45 % and 41 % for *Q. cerris* and *Q. pubescens*, respectively. These values showed the influence of the process conditions, but were well in line with the here determined glucan proportions of ~ 43 % for debarked oak.

Seidel (2019) tested beech under various SE conditions and determined a glucan content of the pretreated material of 39.6 % to 48.25 % and a xylan content of 2.6 to 19.7 %, dependent to the respective pretreatment conditions. Additionally, for acid insoluble lignin values of 25.9 % to 42.9 % were reported and for the acid soluble lignin 4.0 % to 9.9 %, respectively to the pretreatment conditions. Whereas the values for the acid insoluble lignin are in the same range as the values determined in this thesis, the values for the acid soluble lignin are higher. Regarding the sugar contents, higher values were determined here with 62.8 % for glucan, but just 3.3 % for xylan.

Evaluating the results for the AISR, the proportion were increased to the analysis of wood. For the **AISR** this could be traced to the prior mentioned loss of soluble materials during the first disintegration step and represents a relative increase as lignin was not sufficiently removed. Additionally, the condensation of sugar degradation products or tannins could possibly increase the proportion of the AISR, which was reported for the SE process (Aarum et al., 2018; Boussaid et al., 2001; Lomax et al., 1994; van Zandvoort et al., 2013). Interestingly, for oak db and wb the highest AISR values were found with 40 % and 44 %, which could probably relate to the high tannin content of oak species. However, chestnut species are also known for their high tannin content (Mai & Zhang, 2023), but no increased vales were found like for oak. The missing high values for chestnut were probably reasoned in the young age of the trees. Due to the lower age, the tannins were less condensed (Klumpers et al., 1994) and thus potentially dissolved during the SE-process and not included in the SPS.

Robinson et al. (2002) and Frankó et al. (2018) studied the effect of softwood bark and observed increased proportions of acid insoluble lignin and decreased glucan contents, if the bark proportion was increased. These observations were thus in agreement with the here detected effects. However, the authors investigated the effect of wood and bark of coniferous

trees, which are chemically different to the wood and bark of deciduous trees. Cotana et al. (2015) also detected an increased proportion of acid insoluble lignin comparing the initial material with the pulp after SE. Additionally, a correlation between increased severity and increased proportion of this fraction is detected. *Quercus pubescens* with the highest extract content, showed the strongest increase in acid insoluble lignin.

Evaluating the amounts of **ASR** the values for oak and chestnut db and wb with 3 % were slightly higher than for beech and ash with 2 %, but did not notably differ from the values detected in the wood samples. The recovery percentages reported in Table 9, shows recovery values slightly > 100 %. It is assumed that different factors can contribute to this overestimation like instable integration parameters during sugar analyses or that the chosen absorptivity constant was not sufficiently suitable for the determination of the ASR. Further, the sulfuric acid was eventually not completely removed from the AISR or it was not totally dried.

The analysis of the SPS showed, that as expected the main proportion of the hemicelluloses was dissolved and glucan as well as lignin have the dominant share. Additionally, it is indicated that sample with bark show a lower glucan proportion than debarked samples especially for oak, but for beech wb no effect was detected. The high glucan content of chestnut db with 67 % seemed to be favorable in case of potential yield, similar to the indication found for the wood analyses. Further, the proportion of AISR seemed to increase, but it could not be differentiated if this was a relative or real increase. The outputs of this analysis act as reference for following analyses and thus contribute to all research questions.

Table 9: Results of sugar and ASR / AISR analysis of the SPS by acid hydrolysis.

Sample		**AISR**	**ASR**	**Sum** (residues)	**Rha/Man/ Ara/Gal [mg/g]**	**Xyl**	**Glc**	**Sum** (sugars)	**% Recovery** (sugars+lignin) **[%]**
Beech	db	343	23	365	n.d.	33	628	661	103
Ash	db	349	22	371	n.d.	22	654	675	105
Oak	db	404	30	434	n.d.	22	582	604	104
Chestnut	db	349	27	376	n.d.	19	670	690	107
Mix	db	356	22	378	n.d.	29	641	671	105
Beech	wb	348	21	369	n.d.	31	650	682	105
Ash	wb	356	24	380	n.d.	26	574	599	98
Oak	wb	443	32	474	n.d.	17	476	493	97
Chestnut	wb	364	27	391	n.d.	15	576	591	98

n.d. = not detectable AISR = acid insoluble residue ASR = acid soluble residue

db = debarked wb = with bark

5.5 Conversion of cellulose in SPS to glucose by enzymatic hydrolysis

An enzymatic hydrolysis (EH), following the EH scenario 2 was performed to evaluate the conversion rate of cellulose in the SPS to glucose monomers and the resulting yield (Figure 9).

For the **debarked samples** the best conversion rate after 48 h was found for beech with 51.1 %, followed by ash with 47.5 %. In contrast, oak and chestnut showed very low conversion rates of 15.9 % and 15.2 % and the mixed sample was found to have a conversion rate of 34.7 %. The mixed samples includes 60 % beech and 20 % ash and oak, respectively. Considering the performance of the individual tree species, the reduced rates for the mixed sample seemed to be reasoned in the oak proportion.

For the **samples with bark** the picture is similar on the first glance, but for ash and especially oak and chestnut the conversion rate is reduced. In contrast, for beech with bark, similar to the debarked sample a conversion rate of 50.5 % was detected. The conversion rate decrease was found to be strongest for oak with -33 % and chestnut with -14 %.

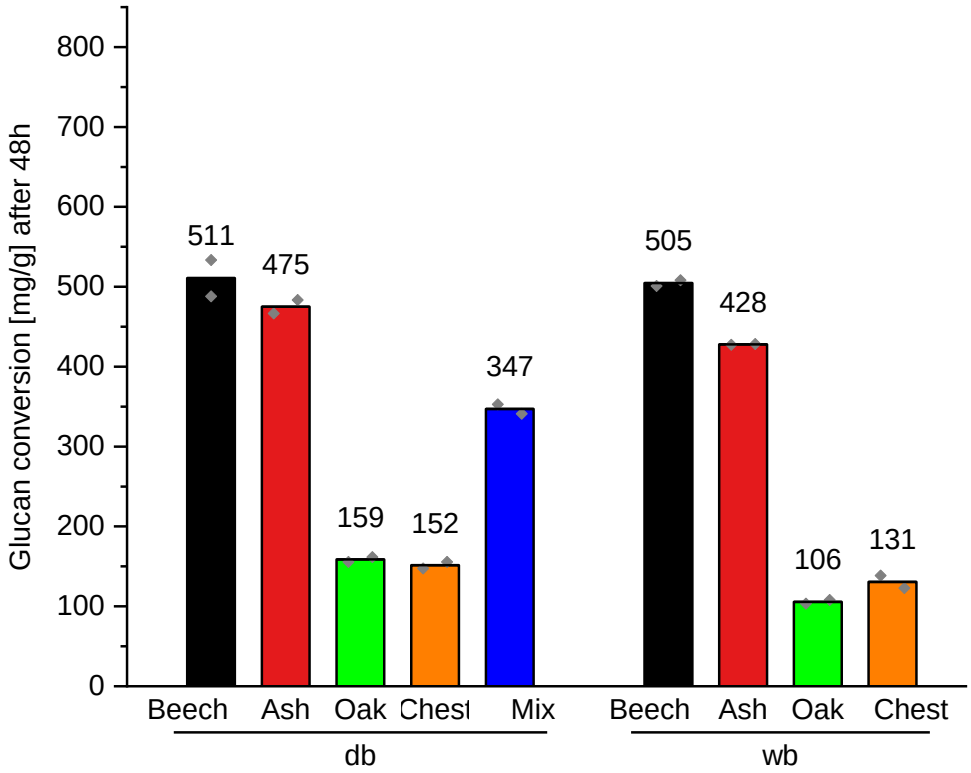


Figure 9: Glc conversion [mg/g] after 48 h; EH with 8 mg protein per g glucan (EH scenario 2); bars refer to the mean values and the symbols mark the individual values of the double determination

Regarding comparison with values form literature, for instance Seidel (2019) determined an enzymatic cellulose digestibility for *F. sylvatica* of 6.9 - 99.1 %, dependent to the different process conditions during the SE pretreatment. For the enzymatic hydrolysis a solid load of 1 % w/w cellulose and an enzyme load of 60 FPU g^{-1} cellulose was applied and the reaction ran at 50 °C for 120 h. Cotana et al. (2015) found an enzymatic hydrolysis yield of 40.5 - 97.82 % for *Q. ilex*, 48.4 - 94.8 % for *Q. cerris* and 42.03 - 96.3 % for *Q. pubescens*. These values were dependent to the respective conditions during SE pretreatment and enzymatic hydrolysis. The enzymatic hydrolysis was performed for 48 h with enzyme loadings from 7 - 13 % based on dry matter. The results of Cotana et al. (2015) are notably higher than

the conversion rates determined in this work. However, these examples from literature show that the values for the conversion rates are strongly dependent on the pretreatment process conditions and the chosen set up for enzymatic hydrolysis, especially regarding time, enzyme and solid load. Therefore, a direct comparison and evaluation of the here determined values and values from literature is not possible without restrictions.

These results clearly show that the conversion by enzymatic hydrolysis was strongly influenced by the tree species. The glucan content determined by acidic hydrolysis did not correlate with the suitability for enzymatic conversion. The influence of bark has to be evaluated in a differentiated way, as there is no indication of a negative influence for beech, but especially for oak it seemed to be strong. These analyses contributed mainly to research question 1.1.

5.6 Monomeric sugars in the liquid process stream (LPS)

To complete the consideration of the sugars within the process streams, the sugar content and composition of the LPS were analyzed. Preliminary tests with acidic hydrolysis of the LPS prior to sugar analysis did not reveal notable amounts of oligomeric sugars (results not shown). This indicated, that the main sugar proportion was available as monomers. In contrast, Kim et al. (2011) detected xylo- and gluco-oligomers in the pretreatment liquid of liquid hot water pretreated maple.

The monomeric sugars were directly analyzed by ion chromatography, which was suited to separate the different monomeric sugars inclusive rhamnose (rha). Rhamnose is a typical hemicelluloses sugar in wood (Fengel & Wegener, 2003), but due to the small proportion it is often not mentioned or cannot be separated and thus not detected by means of common sugar separation methods applying polymer based analytical columns modified with Pb^{2+}.

As reference for the quantitative analysis of the LPS the total dry matter was determined. This prevents distortions through potential concentration variations within the samples, which are based on the process conditions during and after the pretreatment. Table 10 presents the solid matter of the LPS for each species db and wb.

Table 10: Solid matter of LPS samples after filtration and freeze drying; relative deviation < 5 %

Samples db	**Solid matter [mg/ml]**	**Samples wb**	**Solid matter [mg/ml]**
Beech	77.7	Beech	76.9
Ash	65.0	Ash	45.5
Oak	61.1	Oak	65.2
Chestnut	57.6	Chestnut	65.9
Mix	58.7		
db = debarked		wb = with bark	

Based on the solid matter the individual sugar contents are presented in Table 11. The column “Further compounds”, represents the peaks, which appeared in the chromatogram, but could not be aligned to a reference substance and were thus not included in the column “Sum”.

Table 11: Proportion of sugars (shown as monomers) in the LPS; “Sum” excludes the values for “Further compounds”.

Sample		Further compounds	Rha	Man	Ara	Gal	Xyl	Glc	Sum
					[%]				
Beech	db	3.2	3.0	2.7	1.4	2.5	48.1	6.8	64.5
Ash	db	3.2	3.1	4.7	3.1	1.4	45.3	9.9	67.5
Oak	db	2.2	2.8	2.6	1.7	3.0	48.0	8.1	66.2
Chestnut	db	2.0	2.6	4.5	0.7	2.6	44.7	9.0	64.0
Mix	db	4.1	3.1	2.8	1.7	2.6	50.6	6.5	67.2
Beech	wb	4.3	3.4	2.9	2.1	2.7	50.5	6.2	67.7
Ash	wb	7.3	3.4	4.0	3.5	1.0	41.2	4.0	57.2
Oak	wb	2.9	2.8	2.2	2.3	4.8	40.5	9.9	62.5
Chestnut	wb	3.0	3.4	5.2	1.8	3.8	50.1	8.5	72.7

db = debarked wb = with bark

From the here presented results the LPS of the debarked samples covered a range of 64.0 – 67.2 % for the **sugar sum**, but for the samples with bark the values ranged from 57.2 % to 72.7 %. The amount of sugars was notable lower for ash and oak wb, but the sugar content for beech wb and chestnut wb was higher than for the db samples.

Probably, these findings correlated with the bark proportion similar to the evaluation of the sugar content of the SPS. For ash and oak a high bark proportion ~15 % was found and it was reported that bark contained less hemicelluloses than wood (Dietrichs et al., 1978), which could explain the lower values for ash and oak wb. Contrary, beech and chestnut were found to have a small bark proportion, thus the proportion of wood was higher, resulting in a smaller delta. Additionally its was reported that sugars in bark get more easily dissolved than from wood (Dietrichs et al., 1978), which possibly overcompensated this delta. This could explain the similar or slightly higher sugar contents found in the LPS of beech and chestnut db.

Figure 10 shows the **proportions** of the different sugar monomers detected in the samples. As expected, **xylose** takes the main proportion in the range of 45.3 % to 50.6 % for the debarked samples and 40.5 % to 50.5 % for samples with bark. Additionally, some proportions of **glucose** were found, which possible originate from the hemicelluloses or from amorphous cellulose, which was already degraded towards glucose during the SE process. The hemicellulose sugars arabinose (ara), galactose (gal) and rha were detected in small proportions < 5 %

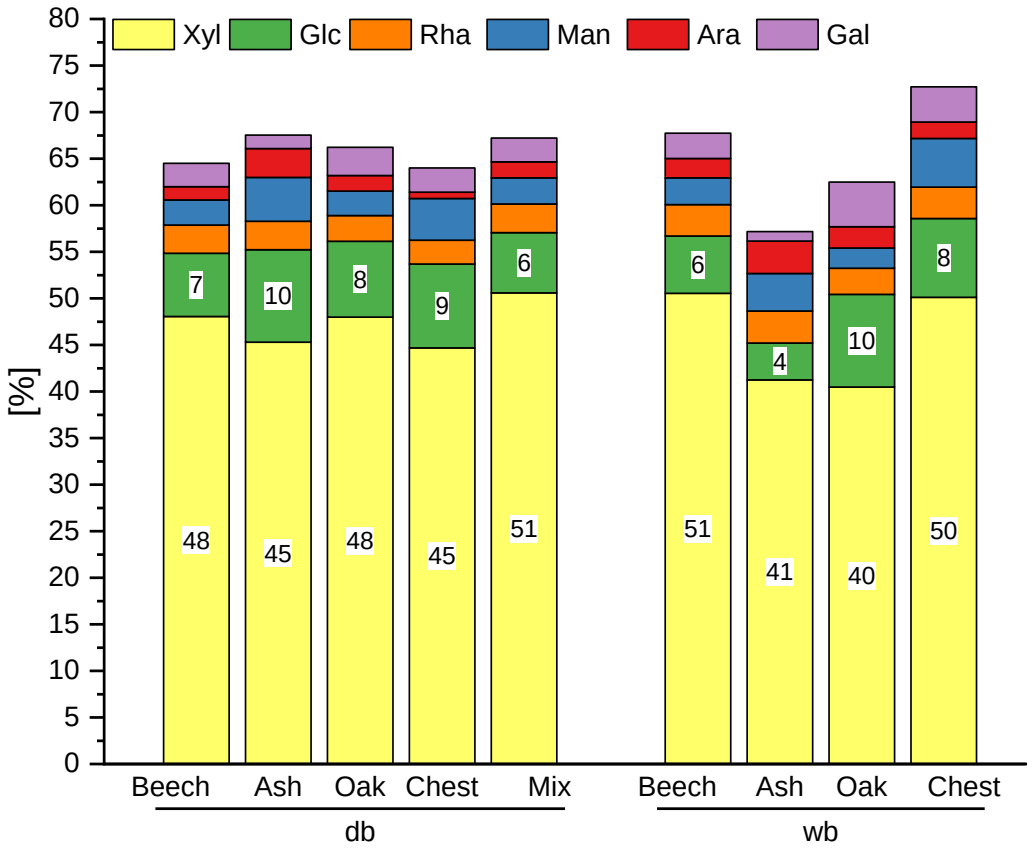


Figure 10: Sugar composition (monomers) of the LPS, based on the solid matter of the fraction

Regarding the sugar proportions, ash wood for example was found to contain less xylan with 15.2 % (db) and 15.5 % (wb) than beech. Interestingly, this discrepancy was also detectable in the sugar contents of the LPS. Ash showed xylose proportions of 45 % (db) and 41 % (wb), and beech showed 48 % (db) and 51 % (wb). The same correlation was observed for mannose for example for ash and chestnut wb, which showed the highest mannose proportions in wood with 2.0 % and parallel the highest proportions in the LPS with 4.0 % and 5.2 %. Additionally, for arabinose the highest values were detected in ash wood and similar in the LPS of ash. This indicated a correlation between the sugar proportions detected in wood and the LPS (under consideration of the applied pretreatment conditions), like a species-specific “sugar fingerprint”.

Interestingly, the xylose content of oak wood db /wb with approximately 17 % ranks in between the values for the other species and this correlates with the xylose proportions of the LPS of oak debarked. However, for oak wb the lowest xylose content is detected, which should be expected to be higher following the grading between the species. One reason could be that for oak wb less xylose dissolved during the SE process, which should be recognizable from a higher xylose content in the SPS. However, the xylan content of the SPS with 1.7 % was quite low compared to the other species. Another potential reason was that the pretreatment severity was performed at an intensity level, where the dissolved xylose was further degraded for example to furfural or further. In bark xylans were reported to have a higher proportion than cellulose, contrary to wood. The content of O-acetyl-4-O-methylglucuronoxylan in oak was found to be even higher than for beech. This theoretical higher volume of xylans, which was easily dissolved and degradable was probably additionally enhanced by the high bark proportion of oak (Dietrichs et al., 1978).

Overall, these analyses showed that the LPS is dominated by monomeric sugars originated from hemicelluloses, under consideration of the chosen process conditions. Further it was indicated that the sugar proportions detected in wood correlated with the respective proportions in the LPS, at least in most cases. These results contributed to the research questions 1.2 and 1.3 as well as 2.2.

5.7 Characterization of wood extractives

In respect to the research questions about species-specific patterns and component distribution the fraction of extractives and other soluble substances became important. The analysis of the raw material worked as reference basis for comparison and to explain relations.

5.7.1 Proportion and solubility of wood extractives

The fraction of wood extractives include various substance classes, which are soluble in different solvents dependent to the polarity and do not belong to the structural components like cellulose, hemicelluloses and lignin. This chapter further excludes the mineral compounds, which will be considered later (chapter 5.9). For a general analysis of the solubility of the extractives within the different tree species a successive Soxhlet extraction with organic solvents (petrol ether, acetone and methanol) was performed. Figure 11 presents the quantitative results of the **organic successive extraction**. It was observed that the main share of the extractives was dissolved within the more polar solvents of acetone and methanol. In contrast, solely a minor proportion was solved in the unpolar solvent petrol ether. These proportions were expected as it is commonly known that hardwoods contain a higher proportion of polar soluble extractives than extractives soluble in unpolar solvents.

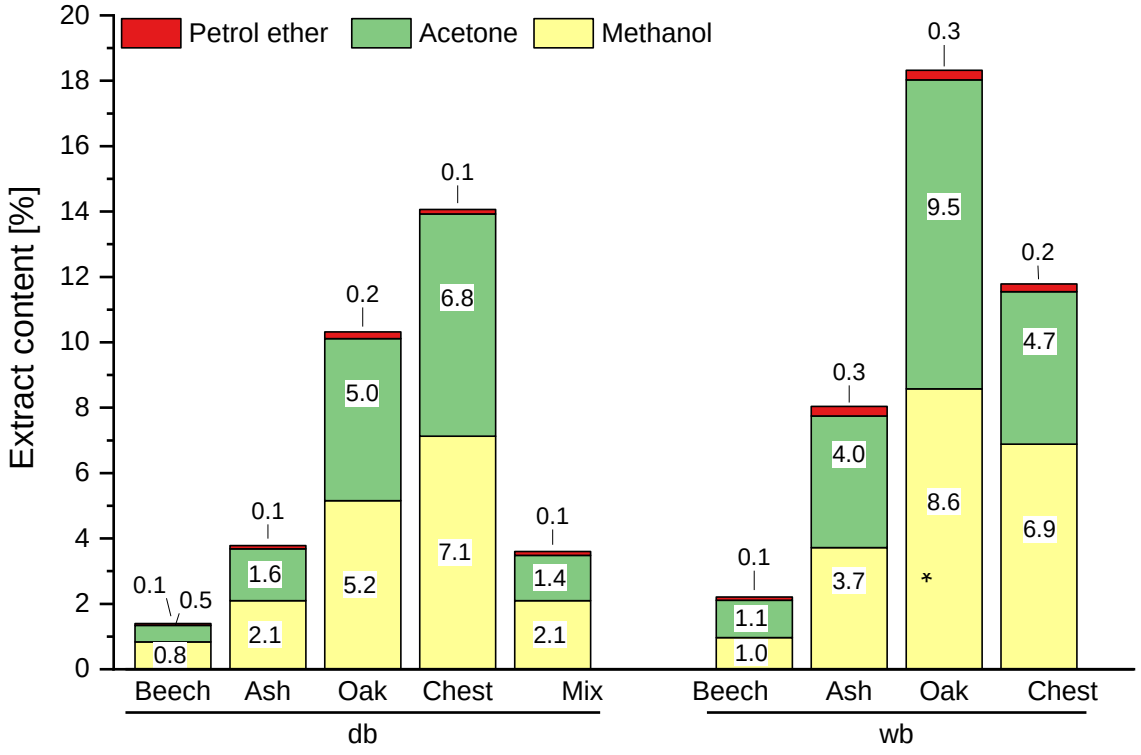


*Figure 11: Extract contents [%] of successive organic solvent extraction; * relative deviation slightly > 10 %.*

The main focus was set on in situ extractions with **hot water.** On the one side, water is also a polar solvent. On the other side, the steam explosion process does not implement organic solvents. Therefore, it was assumed that the fraction, extracted by hot water showed some similarities to the liquid process stream after SE. This assumption and the good solubility of hardwood extracts in polar solvents supported the choice to use the hot water extraction to prepare the samples for sugar analysis, which was presented in chapter 5.7

The quantitative results of the hot water extraction are shown in Figure 12, including one extraction cycle for ash and beech and two cycles for chestnut and oak samples as well as the mix to secure a proper extraction. The total extract contents determined by hot water extraction were notable higher than for the successive extraction. This can be related to the higher polarity of water as solvent and the high proportion of polar soluble extractives of these species. The results of successive and hot water extraction were in line with each other regarding the differences between the species and the general trend.

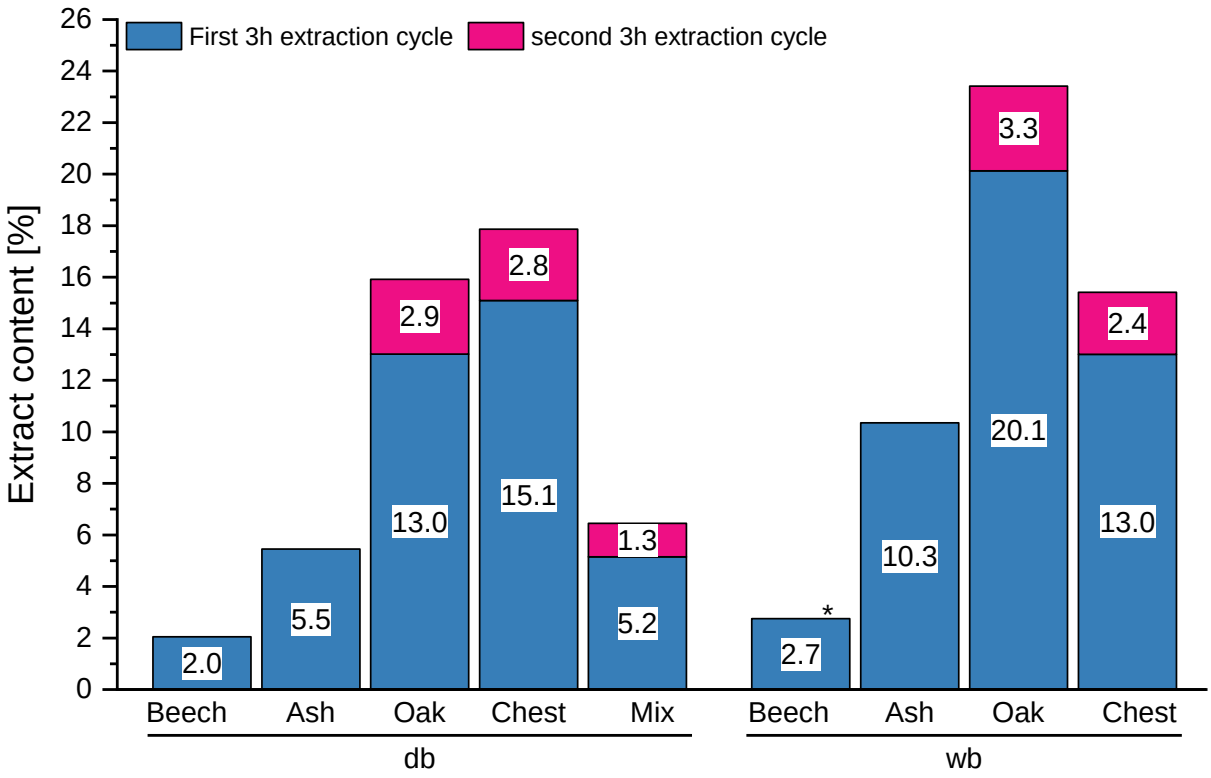


*Figure 12: Extract contents [%] determined by hot water extraction; * relative deviation slightly > 10 %*

The **total extract content** differs strongly between the tree species. For the **debarked samples** the order was beech < ash < mix < oak < chestnut.

It was reported that the typical wood extractives of oak and chestnut polymerize into bigger molecules during the aging of the heartwood and thus become less soluble in water (Klumpers et al., 1994). This would explain the higher extract content of chestnut db compared to oak db. Further, this supported the assumption of the young age of the harvested chestnut trees, which was discussed in chapter 5.1. The value detected for the mixed sample with 6.5 % fitted well in the picture, as the extract content was slightly higher than for beech and ash, but still notably lower than oak. This was related to the share of oak and ash material within the mixed sample, which both accounted to 20 %.

For the samples with bark the order was beech < ash < chestnut < oak. The extract content was found to be higher in samples with bark, except for chestnut. Even under consideration of the young age of the tree resulting in a thin bark and thus probably less impact on the total extract content, the value should be at least similar to the debarked sample. However, the discrepancy remained even after repeated determination. The discrepancy between the results can just be explained by the natural variability of the extract content between individual trees.

In Wagenführ and Scheiber (1974) a range of 9.5 - 15.9 % was reported for the solubility of *C.sativa* in hot water and for *Q.robur/petraea* values with 5.4 - 12.2 % were reported. Additionally, Fengel and Wegener (2003) reported a hot water extract content of 12.2 % for *Q.robur*. With extract content of 17.9 % for chestnut db and 15.9 % oak db the here determined values were higher than the values reported in literature. Next to the natural variability, also differences in the experimental conditions can cause discrepancies between the results. In the here presented case, the values for chestnut and oak after the first extraction cycle with 13.0 % and 15.1 % were in line with the values in literature, but the extraction was extended to ensure an extensive extraction. The values for hot water extract contents reported in literature for *F. excelsior* with 2.9 - 6.8 % (Wagenführ & Scheiber, 1974) agreed with the here determined values of 5.5 %.

Dietrichs et al. (1978) found a hot water extract of 30.0 % for pure beech bark and of 19.5 % for pure oak bark, after 24 h Soxhlet extraction. This supports the observed increased extract contents for samples with bark, which were detected in the experiments and points out the fact, that bark contains higher amounts of extractives than the respective wood (Mai & Zhang, 2023).

These analyses clearly showed that the species differ strongly in their extract contents and that the inclusion of bark increased the total extract proportion. It was further shown that for the included tree species the polar soluble fraction of extractives takes the major proportion. These results contribute to research question 1.3 and 2.2.

5.7.2 GC/MS analysis

To get an overview on the composition of the wood extractives, the individual fractions were analyzed at GC/MS. However, the focus was set on the analyses of the hot water extracts, as already discussed in the previous chapter.

Organic solvent extracts

A qualitative GC / MS analysis of the different extract fractions from the **successive extraction** showed the presence of unsaturated and saturated fatty acids likely from triglycerides as well as steroids in the petrol ether fraction. For instance linoleic acid and oleic acid as unsaturated fatty acids and stearic acid as saturated fatty acids were detected for all four species beech, ash, oak and chestnut (debarked and with bark). ß-Sitosterol and Stigmasta-3,5-dien were detected in all samples db and wb. The steroid campesterol was detected for ash and chestnut db.

For the more polar solvents methanol and acetone mainly sugar alcohols and smaller aromatic compounds were detected, like gallic and ellagic acid as well as fraxinol derivatives or catechin as flavonoid. These substances are characteristic for the different wood species (Fengel & Wegener, 2003). Fraxinol derivatives were observed for ash, but solely in the samples with bark (acetone fraction). Ellagic acid was detected in oak and chestnut db and wb and gallic acid for oak and chestnut db and oak wb. Depending on the polarity, ellagic acid was detected in the acetone and methanol fraction, but gallic acid solely in the methanol fraction. Catechin is a common flavonoid in wood (Mai & Zhang, 2023), but was solely detected in beech samples db and wb in both polar solvent fractions (acetone and methanol). As a mixture out of beech, ash and oak wood proportions (debarked), ellagic acid and catechin were detected for the mixed sample as well.

Hot water extracts

For the analyses of the **hot water extracts** by GC/MS, dry extract was solved in water and separated by SPE. This sample preparation was chosen to remove the solubilized sugar fraction. Pre-test showed that the dissolved sugars were detected with high intensities and frequent peaks at the GC / MS after derivatization. This led to strong overlapping with peaks from other substances. Therefore, the fraction of the hot water extract, which was adsorbed on the SPE phase was eluted and used for the analyses. The proportion of the eluted phase recovered after SPE is presented in Table 12. The recovered proportions ranged between ~ 44.7 % and 64.1 % of the initial amount of hot water extract.

Table 12: Proportion of the eluate of the hot water extract fraction after SPE separation (chapter 4.2.3)

Sample		**Sample**	
db		**wb**	
	[%]		**[%]**
Beech	44.7	Beech	59.2
Ash	64.1	Ash	61.8*
Oak	56.9	Oak	61.1*
Chestnut	53.3	Chestnut	55.9
Mix	57.6		
db = debarked	wb = with bark	* relative deviation > 5%	

During the following GC/MS analyses proportion 3.8 – 8.9 % of the eluted phase could be detected by GC/MS measurements. These substances were mainly identified as typical wood extractives, with similar compounds detected in the polar solvent fractions of the organic successive extraction.

For oak and chestnut the substances gallic acid could be detected with 0.2 % and 1.0 % for the debarked samples and with 0.78 % and 1.44 % for samples with bark. Ellagic acid was detected in higher proportions of 2.8 % and 3.3 % for oak and chestnut db and with 3.0 % and 3.9 % for wb samples. The increased proportions fitted to the already detected higher extractive proportion for samples containing bark. Additionally, it was reported that chestnut contain higher proportions of gallic acid, which is in line with the here presented results. However, it had to be considered, that the quantification of some substances at GC/MS was challenging for instance ellagic acid, likely due to carryovers. This led in some cases to insufficient relative deviations, which are individually marked in Table 13.

The ellagic acid detected in ash, was most likely due to impurities, as this substance is typically not present in ash wood. Further two peaks were identified as catechin, with proportions of 0.7 % and 1.9 % for beech db and wb.

The proportion of the eluted phase of ash db/wb after SPE was the highest and the measurements at GC/MS also showed the highest recoveries. Regarding this tree species mainly coumarin derivatives like fraxinol, as typical extractives of the genus *Fraxinus* were detected with proportions of 1.4 % (sum of both peaks). However, these coumarins were solely detected in the sample with bark, similar to the organic successive extraction. Another substance mainly detected for ash wb, but also db was tyrosol a phenolic compound.

Next to these substances the lignin building blocks coniferyl alcohol and sinapyl alcohol were detected as well as some sugars, which probably originate from glycosides. These were cleaved during the gasification of the sample during injection or the derivatization reaction.

As stated in the beginning, the analyses of the wood extractives mainly function as reference basis for the characterization of the LPS in the following chapters. These analyses, thus contribute to research questions 1.2 and 1.3 as well as 2.2.

Table 13: GC/MS analysis of silylated eluate of the hot water extracts after SPE separation

Retention time [min]	Proportion [%] based on eluate mass									Substance
	debarked					with bark				
	Beech	Ash	Oak	Chest	Mix	Beech	Ash	Oak*	Chest	
10.02	---	1.11	< 0,14	---	0.20	---	0.72	---	---	Phenol -CH_2-CH_2OH (Tyrosol)
11.40	< 0,08	0.34	< 0.14	< 0.16	0.11	< 0.22	0.39	< 0.15	---	Sugar
11.97	0.13	1.55	0.82	< 0.16	1.05	< 0.22	2.24	1.04	< 0.20	Sugar
12.87	0.15	< 0.15	< 0.14	< 0.16	< 0.11	0.28	---	< 0.15	< 0.20	n.i.
13.22	< 0,08	0.37	---	---	< 0.11	< 0.22	0.38	< 0.15	---	n.i.
13.30	0.33	< 0.15	0.11	< 0.16	0.17	0.49	< 0.15	0.16	< 0.20	n.i.
13.95	---	---	0.23	1.01	0.15	---	---	0.78	1.44	Gallic acid
13.99	0.52	0.61	---	---	0.18	0.55	0.43	---	---	Guaiacyl -CH=CH-CH_2OH (Coniferyl alcohol)
15.34	0.17	< 0.15	< 0.14	---	< 0.11	< 0.22	---	---	---	Syringyl -CH=CH-CH_2OH (Sinapyl alcohol)
15.34	---	---	---	---		---	0.22	---	---	n.i.
15.50	0.17	0.44	0.32	0.46	0.27	0.21	0.41	0.25	0.44	Hydrocarbon residue
15.96	---	---	---	---	---	---	0.26	---	---	Fraxinol / Fraxidin / Isofraxidin
16.21	---	---	---	---	---	---	0.78	---	---	Fraxinol / Fraxidin / Isofraxidin
18.20	< 0.08	0.19	0.11	< 0.16	0.15	< 0.22	< 0.15	< 0.15	< 0.20	n.i.
18.32	---	---	---	---	---	---	---	---	---	Internal standard
19.08	0.22	0.26	< 0.14	---	0.16	< 0,22	< 0,15	< 0.15	< 0.20	Guaiacyl - derivative
19.20	0.39	0.37	< 0.14	< 0.16	0.23	0.26	< 0.15	< 0.15	< 0.20	Guaiacyl - derivative
19.95	0.54	< 0.15	---	---	< 0.11	0.35	< 0.15	---	---	n.i.
20.27	0.18	---	---	---	---	0.41	< 0.15	---	---	n.i.
20.41	0.35	< 0.15	---	---	< 0.11	0.30	< 0.15	---	---	n.i.
20.48	---	1.16	---	---	0.15	---	1.52	---	---	2-O-(2-(4-Hydroxyphenyl)-ethyl)-glycoside
21.08	< 0.08	---	0.34	0.28	0.15	---	---	0.44	0.31	n.i.
21.19	0.20	---	---	---	---	0.44	---	---	---	Flavonoid (Catechin)
21.34	0.46	---	---	---	< 0.11	1.47	---	---	---	Flavonoid (Catechin
21.48	---	---	---	---	---	0.33	---	---	---	n.i.
21.73	---	---	---	---	---	1.04	---	---	---	n.i.
22.21	---	---	0.32	---	0.13	---	---	---	---	n.i
22.46	---	0.25	---	---	< 0.11	---	0.41	---	---	n.i.
23.58	---	---	---	---	---	---	1.16	---	---	n.i.
23.68	---	0.25	---	---	< 0.11	---	< 0.15	---	---	n.i.
24.29	---	< 0.15	2.75	3.28	2.22	---	---	3.00	3.92	Ellagic acid
Sum	3.83	6.89	4.99	5.03	5.31	6.11	8.90	5.67	6.11	
	relative deviation > 10%			* single determination		n.i. not clearly identified			---	Peak not detected

5.7.3 pH values (hot water extracts)

To characterize the materials further, the pH was measured in the hot water extract, after cooling to room temperature. The respective values are summarized in Table 14. As to be expected the species oak and chestnut show more acidic pH values ≤ 4.0 than beech and ash with values ≥ 5.0.

For *F. sylvatica* and *Q. robur* literature reported pH values of 5.3 and 4.8 for hot water extracts from wood and for bark pH values of 5.0 and 3.9 were reported. Other sources gave pH values of wood with 5.4 and 5.8 for *F. sylvatica* and *F. excelsior* as well as 3.9 for *Q. petraea* and a pH < 4 for *C. sativa,* but without defining the measurement method further (Fengel & Wegener, 2003; Richter & Ehmcke, 2018; Wagenführ & Scheiber, 1974) The here determined pH values thus matched the values reported in literature. However, the pH value seemed to be strongly dependent on the wood species, which is reasoned in the different composition of wood extractives. For example for oak and chestnut the high content of tannins (Mai & Zhang, 2023) probably implements high proportions of related acids, which decreased the pH of the hot water extracts. The lower pH values for hot water extracts from bark, reported in literature, could not be detected here, which was likely due to the low bark content of the samples with bark.

Table 14: pH values measured in cooled hot water extract

Sample db	**pH**	**Sample wb**	**pH**
Beech	5.7	Beech	5.6
Ash	5.0	Ash	5.0
Oak	3.7	Oak	3.9
Chestnut	4.0	Chestnut	4.0
Mix	5.1		
db = debarked	wb = with bark		

5.7.4 FTIR analysis of hot water extracts

The freeze dried hot water extracts were analyzed by FTIR to get an impression on the chemical structure of the extracted fraction. Interestingly the spectra of beech and ash appeared with a similar absorption pattern and the same accounted for oak and chestnut. However, between beech/ash and oak/chestnut strong differences were detected in the absorption spectra. Figure 13 shows exemplary the spectra of the hot water extracts of beech db and oak db.

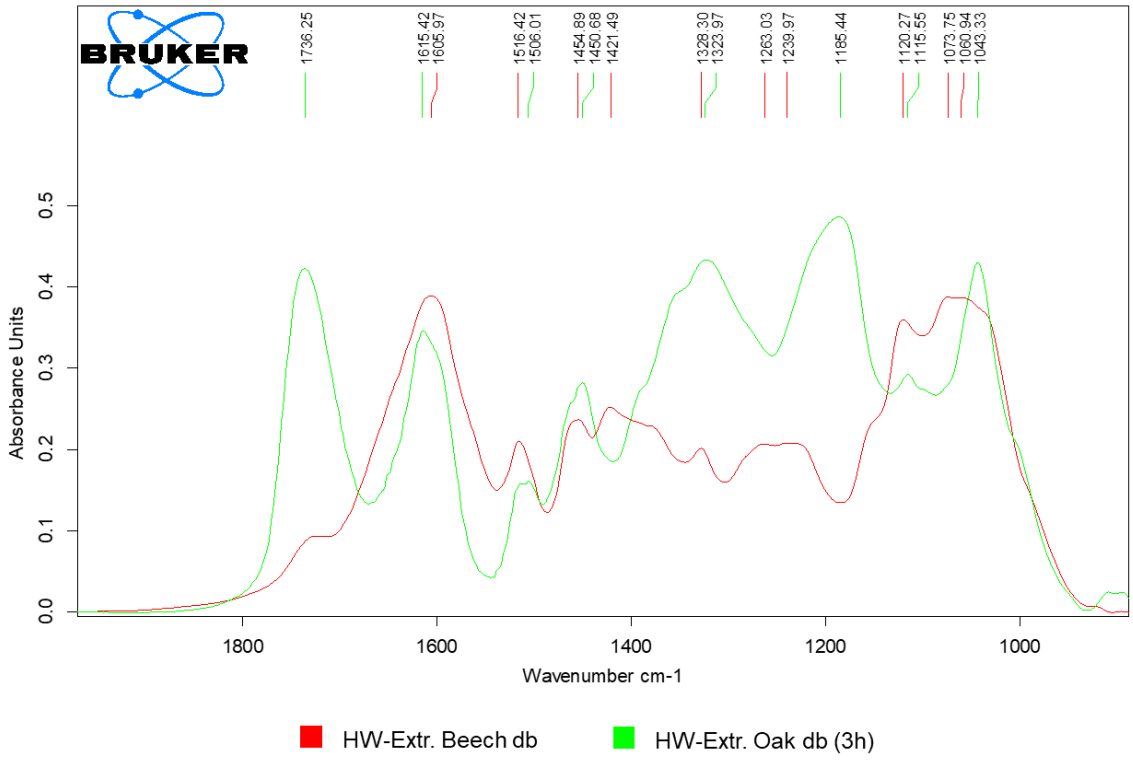


Figure 13: Representative FTIR spectra (fingerprint region 1800 – 800 cm^{-1}) of hot water extracts of beech and oak debarked (baseline corrected)

The most notable difference is in the absorption area around 1730 cm^{-1}, where oak showed a strong peak, which was solely present as a shoulder in the spectra of beech. For the area between 1730 – 1725 cm^{-1} the signal belongs to C=O valence vibrations of acetyl- or COOH groups and between 1738 – 1709 cm^{-1} the signals are due to C=O stretch. These signals can be allocated to unconjugated ketons as well as carbonyl groups. Additionally, these signals are typical for ester groups, which likely origin form carbohydrates (Schwanninger et al., 2004). For oak and chestnut it is commonly known, that these species contain hydrolyzed tannins. These are based on gallic acid units, which react by esterification with glucose or other gallic acid units and oxidative coupling reactions towards gallotannins and ellagitannins (McManus et al., 1985; Niemetz & Gross, 2005). Therefore, the strong signal around 1730 cm^{-1} found for oak and respectively for chestnut was probably reasoned in the C=O signals from esters or carboxyl groups of dissolved gallo- or ellagtannins. Goriparti et al. (2013) (support information) assigned the detected IR signal at 1725 cm^{-1} during the analysis of ellagic acid towards C=O stretching, which thus has to be assigned to the ester bonds within the molecule (Goriparti et al., 2013).

Further, the spectra showed one strong signal in the area of 1600 cm^{-1} and a smaller absorption signal around 1500 cm^{-1}, which are assigned to aromatic structures, which could be probably related to dissolved lignin or tannin structures.

The signal around 1324 cm^{-1} was reported to mark the presence of S-lignin units (Faix, 1991; Schwanninger et al., 2004). As the absorption signal in this region was notable stronger for oak, this could indicate, that the proportion of soluble S-lignin was higher in oak than in beech and thus the content was higher in the hot water extract. However, the signal was broad and thus probably several signals overlapped, which could also originated from hemicelluloses or cellulose (Gierlinger et al., 2008).

Goriparti et al. (2013) (support information) related the peak at 1190 cm^{-1} detected during IR measurements of ellagic acid to the vibration of ester bonds. This could be possibly transferred to the here detected strong signal at 1185 cm^{-1} in the spectra of oak and chestnut respectively. This would support the assignment of the signal at 1736 cm^{-1} towards ester groups within dissolved tannins. Further, the signals around 1731 – 1704 cm^{-1} and 1325 – 1317 cm^{-1} are described as characteristic bands for hydrolyzed tannins (Falcão & Araújo, 2013)

The signals around 1120 cm^{-1} can be assigned to C-C and C-O stretching and signals between 1060 – 1025 cm^{-1} belong to C-O valence vibrations (Schwanninger et al., 2004). This would fit to the signals found for beech at 1120 cm^{-1} and the broad signal around 1060 cm^{-1} and could probably originate from sugar proportions dissolved in the hot water extract. In the spectra of oak a small signal was detected at 1115 cm^{-1}, similar to beech, but there is no broad signal around 1060 cm^{-1}, but a more defined peak at 1043 cm^{-1}. This absorption region was assigned to alkyl C-ether vibrations reported in Schwanninger et al. (2004) and Goriparti et al. (2013) assigned the signal at 1052 cm^{-1} detected for ellagic acid towards ester linkages, like the signals at 1190 cm^{-1}.

5.8 Extractives and degradation products in the liquid process stream (LPS)

The LPS was already analyzed for dissolved monomeric sugars, but following literature (Shafiei et al., 2015) this process stream should further contain sugar and lignin degradation products. Additionally, based on the results of the hot water extract analyses, it was expected to contain some proportions of wood extractives. Besides sugar analyses (chapter 5.6) the LPS was thus analyzed further after separation of fine solids. It was assumed that the fine solids were residuals from the SPS, which were not properly separated from the LPS. A screening of the solid fraction by pyrolysis GC/MS (Method D) supported this assumption. The pyrolysis GC/MS analyses detected mainly the substance levoglucosan, which is a common pyrolysis product of hexoses (Fengel & Wegener, 2003; Heigenmoser et al., 2013). Additionally, various guaiacyl- and syringyl- units with different side chains or functional groups as well as some not clearly identifiable hydrocarbon residues were detected. This strongly indicated the presence of lignin and cellulose, which were the main components of the SPS. Therefore, for the following description of the results, the term LPS solely refers to the liquid fraction of the process stream.

For the composition analysis of the LPS regarding degradation products and potential extractives, different approaches were used. The first approach comprised the analysis of the liquid fraction for sugar degradation products, which was performed by HPLC analyses. The second approach applied two different extraction methods to further separate the liquid fraction and reduce the complexity of the individual fractions for analyses at GC/MS.

5.8.1 Analyses of the LPS at HPLC

For the analyses of sugar degradation products by HPLC, the focus was set on acetic acid, levulinic acid, HMF and furfural. Overall, it has to be considered, that the chromatograms of the different samples included a high quantity of different peaks, but these could not be identified. However, this pointed out the high complexity of the chemical composition of this process stream.

The results are shown as percentage of the solid matter of the liquid fraction, similar to the results of the monomeric sugars (chapter 5.6). The **total proportion** (see Table 15) of the detected and identified sugar degradation products ranged for the debarked samples between 12.1 % and 8.0 % and for the samples with bark between 14.2 % and 8.4 %. The value for the mixed sample was detected with 11.0 % and thus laid between beech/ash and oak/chestnut, which fitted to the sample composition (60 % beech, 20 % ash and oak). Interestingly, oak and chestnut db and wb showed lower total proportions than beech and ash. Additionally, the total proportion of samples with bark was slightly increased compared to the debarked samples.

Table 15: Detected proportions of sugar degradation products in the LPS based on the solid matter (chapter 5.6).

Sample		Acetic acid	Levulinic acid	HMF	Furfural	SUM
				[%]		
Beech	db	8.96	2.69	0.11	0.30	12.06
Ash	db	7.93	2.62	0.18	0.23	10.96
Oak	db	5.33	2.26	0.17	0.27	8.03
Chest	db	5.87	2.10	0.20	0.27	8.45
Mix	db	8.11	2.53	0.14	0.25	11.04
Beech	wb	9.00	2.73	0.14	0.33	12.20
Ash	wb	11.41	2.26	0.16	0.35	14.18
Oak	wb	5.70	2.08	0.27	0.31	8.36
Chest	wb	6.61	2.14	0.23	0.26	9.25
db = debarked		wb = with bark				

Regarding the proportions of the individual substances (Figure 14), **acetic acid** took the major proportion in all cases. Acetic acid is a degradation product of the hemicelluloses. The acetyl groups, which are allocated at the hemicelluloses, become easily dissolved and form acetic acid under SE conditions (Berg & Guzmán, 2023). The content was found to be highest for beech, ash and the mixed sample with > 7 %. Especially, ash wb showed a high acetic acid content with 11.4 %. In contrast, the values for oak and chestnut were < 7 %. Overall, the samples with bark show a higher content of acetic acid than the debarked samples. This agreed with literature, which reported higher xylan contents in bark compared to the wood (Dietrichs et al., 1978).

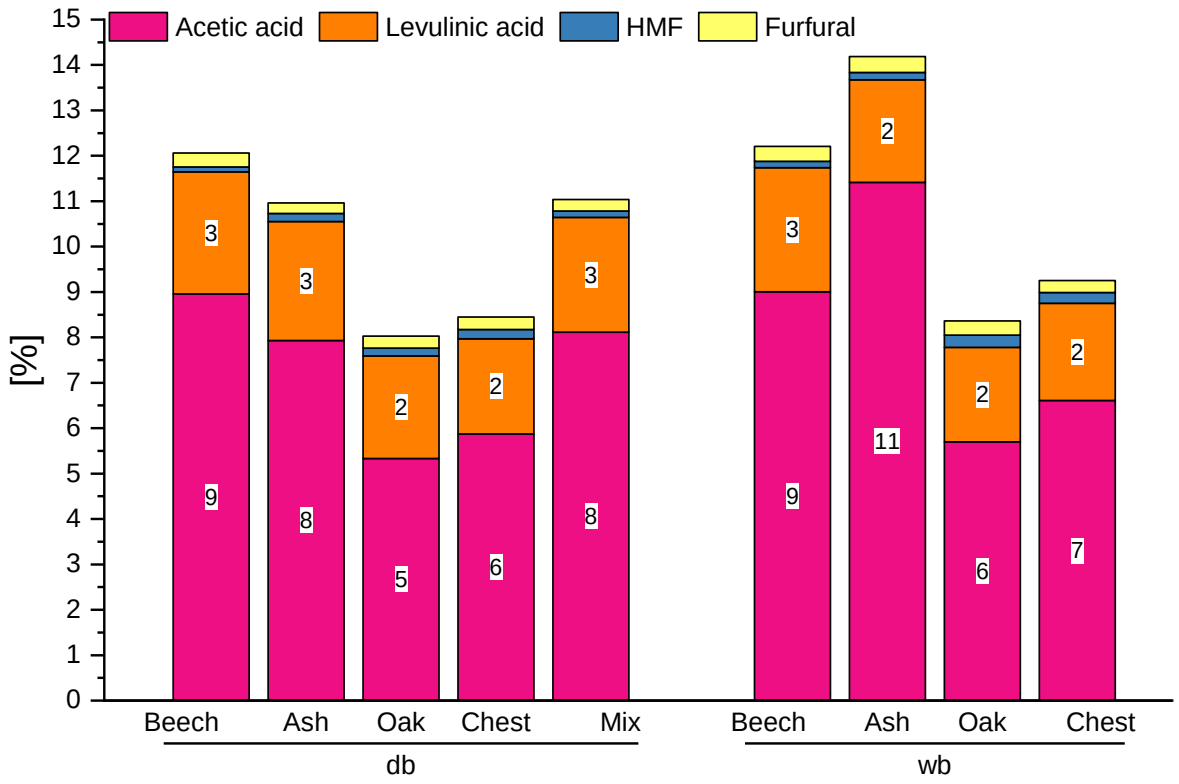


Figure 14: Proportions of sugar degradation products analyzed by HPLC; values based on the solid matter of the LPS

The second highest proportion was shared by **levulinic acid**, which showed proportions of 2 – 3 % over all samples. For the values of levulinic acid it has to be considered, that the peak could not be separated perfectly from a neighboring peak, thus the values can be compared between the measurements, but should not be seen as absolute values. Interestingly levulinic acid is a secondary sugar degradation product as it derives from **hydroxymethylfurfural (HMF)**, which is formed as a degradation product of C_6 sugars (Berg & Guzmán, 2023).

The proportions of HMF and **furfural**, the latter one is a sugar degradation product of C_5 sugars, were low < 1 %, but samples with bark showed a slightly higher content than the debarked samples. Additionally, the proportion of furfural was found to be slightly higher than that of HMF.

On the first impression, the low content of sugar degradation products was seen beneficial because it indicated low losses of the target products (C_6 sugars). However, the presence of levulinic acid as the degradation product of HMF relativized this assumption, because this potentially indicates that the proportion of HMF is reduced, because of too severe conditions entailing a further degradation of HMF towards levulinic acid. Further, substances like HMF, furfural and acetic acid have a relatively low boiling temperature (at atmospheric pressure) with ~ 118 °C (acetic acid), ~ 110 °C (HMF) and ~ 162 °C (furfural) (ECHA, 2023). Therefore, it has to be considered, that the conditions during steam explosion likely led to an evaporation of some proportions of these substances, which condensate separately at collection points within the machinery. This vapor fraction was not included in the analyses and thus a valid evaluation regarding the total volume of sugar degradation products was not possible at this rate.

Negro et al. (2003) detected acetic acid proportions of 0.9 - 2.9 g/100 g poplar biomass dependent on the process conditions during SE of poplar. Interestingly, next to acetic acid also formic acid was detected, which could be not detected in the here presented analyses.

Additionally, furfural and HMF were detected with values < 1 g/100 g poplar biomass, dependent on the process intensity, but the proportion increased with increasing intensity. The comparison of values from literature with the here presented results was difficult, as the concentration of the substances is strongly dependent on the pretreatment process, the material and the intensity. Kim et al. (2011) found 13.1 g/l acetic acid as well as 4.1 g/l furans (HMF, furfural) in the liquid fraction of pretreated maple (liquid water pretreatment). If compared to the here presented results, despite the high variability and various influencing factors, the values of beech db for acetic acid with 6.96 g/l and 0.09 g/l for HMF as well as 0.23 g/l for furfural were notably lower.

These analyses showed the presence of sugar degradation products in the LPS. Acetic acid had the major proportion and similarly showed the greatest proportion variations between the species. These analyses contributed to the research questions 1.2 and 1.3 as well as 2.2.

5.8.2 Component analysis of the LPS at GC/MS

The second approach for the analyses of degradation products as well as extractives in the LPS comprised **two different extraction methods** to further separate the liquid fraction and reduce the complexity of the individual fractions for analyses at GC/MS.

Solid phase extraction (SPE)

The first pre-fractionation was performed by **solid phase extraction** (SPE), similar to the measurements of the hot water extracts. This method was chosen to especially separate the high sugar proportion, which was detected in the LPS (chapter 5.6). However, it has to be considered, that this method also excluded other polar substances like low molecular organic acids, because these were not separated from the sugar fraction during SPE.

The **eluate proportions** recovered after elution of the SPE phase with ACN/H_2O ranges from 5.7 % to 10.5 % with beech and ash showing the lowest and oak and chestnut the highest proportions. The individual values are presented in Table 16 and were calculated based on the solid matter of the LPS (chapter 5.6). Interestingly, the presence of bark showed no clear influence regarding the eluate proportion. However compared to the eluate proportion of the hot water extracts (Table 12) the proportions were much smaller for the LPS, which was likely due to the high sugar content and was thus a relative effect (chapter 5.6).

Table 16: Proportion of the eluted phase of the filtered LPS after SPE separation of the LPS, based on the solid matter of the LPS (chapter 5.6).

Samples db	**[%l]**	**Samples wb**	**[%]**
Beech	5.7	Beech	6.0
Ash	7.1	Ash	7.7
Oak	10.3	Oak	9.2
Chestnut	10.5	Chestnut	10.5
Mix	6.7		
db = debarked		db = debarked	* relative deviation > 5%
Values are calculated based on the solid matter of the LPS			

The mass proportions of the **individual substances** are presented in detail in Table 17. An exemplary chromatogram of the GC/MS analyses of oak wb is included in the appendix (Figure 51, page 192). Overall, with the analysis on GC/MS between 3.8 - 7.5 % of the eluate proportion could be recovered, which equals 0.2 - 0.4 % of the total LPS (dry). Therefore, the here presented results just accounted to a small proportion of the total sample, but still showed some interesting contents.

There were four peaks detected, which could be identified and were **present in all tree species** db and wb. The first substance was guaiacyl-CHO also referred to as vanillin, which was determined with proportions of 0.01 - 0.02 % of the dry LPS. The second substance was syringyl-CHO (syringaldehyde), which accounted to 0.02 - 0.04 % of the dry LPS. The third one was syringyl-COOH or syringic acid. Syringaldehyde and vanillin are the corresponding aldehydes and syringic acid the corresponding acid originated from the guaiacyl- and syringyl-units and thus presented common degradation products of lignin (Fengel & Wegener, 2003). The fourth substance detected in all samples was an extractive and belongs to the substance class of lignans and is called syringaresinol. This substance was determined in proportions of 0.02 - 0.03 %, with beech db showing the lowest and ash wb the highest contents.

Regarding substances found in **individual tree species**, a very interesting substance was found in the LPS of oak db/wb, as well as the mixed sample, which was the lignan lyoniresinol with proportion of 0.02 – 0.03 %. Interestingly, the lignans syringaresinol and lyoniresinol detected here, are seen as equivalent to pinoresinol and isolariciresinol, which are present in softwoods (Mai & Zhang, 2023). Further, for oak and chestnut db/wb and the mix sample, containing 20 % oak, the substance ellagic acid was found in notable amounts of 0.02 - 0.1 %. Additionally, gallic acid was found in oak and chestnut with a proportion of ~ 0.01 %.

Next to the common extractives of oak or chestnut, also a typical extractive of ash was detected: the fraxinol. However, fraxinol was solely detected in the LPS of ash with bark, which is in line with the findings for the wood extractives. Fraxinol was detected in a proportion of ~ 0.03 % and belongs to the substance class of coumarins. This substance class is commonly found in *Fraxinus* species, especially in the bark (Drosky et al., 2014; Kostova & Iossifova, 2007). Another substance just present in the LPS of ash db/wb and the mixed sample (20 % ash) is tyrosol, a phenol with an aligned ethanol group (Karković Marković et al., 2019).

Further, some additional substances were detected, which could not be clearly identified, but possibly have utilization potentials. However, different examination methods would be necessary to identify these substances.

DCM extraction

The second extraction method was a **liquid/liquid extraction with DCM**. In the DCM extracts, many of the substances were present, which were already detected after SPE extraction. Table 18 summarizes the individual substance proportions based on the solid matter of the LPS. An exemplary chromatogram of the GC/MS measurement for beech wb is included in the appendix (Figure 52, page 193). The recovered fraction by GC/MS ranged between 0.6 - 0.8 % based on dry LPS, thus the recovery proportion was found to be higher as for the approach with SPE. However, it has to be considered that this could be due to the lower polarity and a smaller range of dissolved substances and thus the ratio of identifiable substances was higher.

Ellagic acid, gallic acid or the lignan lyoniresinol were not present in the DCM fraction. This can be explained by the polarity differences of the used solvent. For example, gallic acid and ellagic acid were found in the acetone and methanol extracts of wood, but not within the petrol ether fraction. This showed, that these substances are not soluble within unpolar solvents like DCM and this obviously also applies to lyoniresinol. Additionally, gallic acid and

hexahydroxydiphenic acid are often esterified with glucose, thus it is possible, that these substances are glycosides within the LPS, which became cleaved during the derivatization or the measurement procedure at the GC/MS. This would also support the detection of ellagic acid as this substance derives from the hydrolysis of ellagitannins (Mai & Zhang, 2023; Niemetz & Gross, 2005). Additionally, it was reported that lignans frequently form glycosides (Sticher et al., 2015), which supports the absence in the DCM phase.

In contrast, the extractives of ash, the tyrosol and fraxinol were detected in the DCM phase. This strongly indicated that these substances were present as individual substances and not as glycosides. Additionally, the DCM liquid/liquid extractions resulted in better recovery rates of these substances with 0.04 % for tyrosol and 0.07 % for fraxinol, compared to the method with SPE separation. The recovery of syringaresinol could be increased with this separation method as well, presenting values between 0.02 % and 0.07 %. The highest total recovery proportion was repeatedly detected for ash with bark. Compared to the SPE extraction. the only additional and clearly identified components found in the DCM phase were 2-furoic acid and the sugar degradation product HMF.

The quantification of the substances in the DCM phase was done in relation to an internal standard resulting in probably more inaccurate values as the same internal standard was used for all substances independent to the respective response factors. For the quantification of the SPE eluates, an external calibration with three different substances was used to take into account the different response factors of the substance classes. Additionally, an internal standard was used to level measurement fluctuations, by taking the peak ratio of the respective substance with the reference substance of known concentration for calibration (see chapter 4.2.5).

Overall, compared to the GC/MS analyses of the hot water extracts from wood, most of the species-specific wood extractives were also detected in the LPS. However, the sugar and lignin degradation products detected in the LPS, were not present in the hot water extracts. These substances were present due to the impact of the SE pretreatment. These results contribute to research question 1.2 and 1.3 as well as 2.2.

Table 17: Results of the LPS analyses at GC/MS after SPE; green color marks the four peaks detected in every sample.

Retention time [min]	Proportion of the substances [%] db Beech	Ash	Oak	Chest	Mix	wb Beech	Ash	Oak	Chest	Substances
9.63	0.011	0.014	0.016	0.018	0.013	0.011	0.019	0.014	0.014	Guaiacyl - CHO (Vanillin)
9.82	---	0.014*	---	---	0.011	---	0.015*	---	---	Phenol -CH_2-CH_2OH (Tyrosol)
11.19	0.009	0.010	0.015	0.017	0.011	0.009	0.015	0.013	0.014	Sugar
11.5	0.024	0.029	0.027	0.028	0.027	0.026	0.039	0.017	0.020	Syringyl -CHO (Syringaldehyde)
11.73	0.009	0.010	0.015	0.017	0.011	0.009	0.015	0.013	0.014	Sugar
11.99	0.009	0.010	0.014	---	0.011	0.009	0.014	0.013	0.013	Guaiacyl -COOH (Vanillic acid or isomer)
12.14	0.010	0.010	0.014	---	0.011	0.008	---	0.014	0.014	n.i.
12.5	0.008	0.000	0.015	0.016	0.011	0.009	---	0.015	0.014	Guaiacyl -CH_2-CH_2OH
13.27	0.010	0.011	0.014	0.015	0.011	0.009	0.014	0.013	0.013	n.i.
13.36	0.011	0.013	0.018	0.017	0.014	0.012	0.017	0.014	0.015	Syringyl -COOH (Syringic acid)
13.74	---	---	0.013	0.015	---	---	---	0.013	0.013	Gallic acid
14.62	0.008	0.010	0.013	0.014	0.011	0.009	0.014	---	---	Syringyl -CH=CH-CHO (Sinapaldehyde)
14.82	0.008	0.010	0.013	0.014	0.011	0.008	---	---	0.012	n.i.
14.98	0.012	0.011	0.015	0.017	0.013	0.011	---	0.019	0.019	n.i.
15.03	---	0.010*	0.017	0.017*	0.011	0.008*	---	0.012	0.012	n.i.
16.12	---	---	---	---	---	---	0.033	---	---	Fraxinol / Fraxidin / Isofraxidin
16.28	0.022	0.020	0.024	0.025	0.021	0.022	0.014	0.030	0.031	n.i.
18.05	---	---	---	---	---	0.008	---	0.013	0.013	n.i.
18.75	---	---	---	---	---	---	---	---	---	Internal Standard
20.04	---	---	0.025	0.028	---	---	---	0.027	0.026	n.i.
20.29	---	0.018	---	---	0.010	---	0.016	---	---	Lignan (n.i.)
20.7	---	---	0.027	---	0.017	---	---	0.027	---	Lignan (Lyonisresinol)
21.85	---	---	0.075*	0.118*	0.023	---	---	0.088*	0.106	Ellagic acid
23.08	0.017	0.022	0.028	0.029	0.023	0.020	0.033	0.023	0.024	Lignan (Syringaresinol)
SUM LPS	0.17	0.22	0.40	0.41	0.27	0.19	0.26	0.38	0.39	
SUM eluates	2.97	3.15	3.85	3.88	4.07	3.12	3.39	4.10	3.71	

Table shows the proportion of the substances based on the solid matter of the LPS; row "SUM eluates" shows the total proportion based on the eluate
db = debarked wb = with bark * relative deviation > 5% n.i. = not clearly identified

Table 18: Components detected in the LPS after DCM extraction, based on dry LPS; table is continued on the next page

Ret.time	Proportion of the substances [%]									Substances
	db					wb				
	Beech	Ash	Oak	Chest	Mix	Beech	Ash	Oak	Chest	
9.59	✓	✓	✓	✓	✓	✓	✓	0.007	✓	2-Furoic acid
12.25	0.060	0.118	0.133	0.107	0.075	0.066	0.086	0.193	0.164	5-Hydroxymethylfurfural
15.13	0.049	0.064	0.045	0.048	0.048	0.045	0.083	0.027	0.038	Guaiacyl - CHO (Vanillin)
15.35	---	0.039	---	---	✓	---	0.041	---	---	Phenol -CH_2-CH_2OH (Tyrosol)
15.48	---	---	✓	---	---	---	---	✓	✓	n.i.
16.29	---	✓	✓	✓	---	---	---	✓	0.008	n.i.
16.91	---	---	---	---	---	---	0.011	---	---	Guaiacyl -CH_2-CH_2OH
17.03	0.180	0.193	0.144	0.126	0.172	0.168	0.270	0.066	0.094	Syringyl -CHO (Syringaldehyde)
17.13	✓	✓	0.009	0.009	✓	✓	✓	✓	0.008	n.i
17.47	0.010	---	0.011	✓	✓	---	---	✓	0.008	n.i.
17.55	0.007	0.011	✓	✓	✓	0.008	0.013	0.008	0.010	Guaiacyl -COOH (Vanillic acid o. Isomere)
17.70	0.007	0.023	0.010	0.025	0.008	0.020	0.016	0.022	0.013	n.i.
17.86	0.003	✓	0.008	✓	✓	✓	---	0.007	0.010	n.i.
18.06	0.008	---	0.018	0.020	0.008	0.009	✓	0.026	0.027	Guaiacyl -CH_2-CH_2-CH_2OH
18.50	0.009	---	0.010	---	✓	---	---	✓	✓	n.i.
18.57	0.014	0.029	0.016	0.026	0.010	0.013	0.032	0.008	0.015	n.i.
18.64	✓	0.009	✓	✓	✓	✓	✓	✓	✓	n.i.
18.84	0.006	0.016	✓	0.015	0.005	0.016	0.012	0.012	0.008	n.i.
18.90	0.029	0.026	0.035	0.018	0.027	0.022	0.027	0.016	0.019	Syringyl -COOH (Syringic acid)
19.15	0.006	0.009	✓	✓	✓	✓	✓	✓	✓	n.i.
19.34	0.038	0.043	0.069	0.067	0.038	0.033	✓	0.069	0.083	n.i.
19.42	0.006	---	0.013	0.014	✓	0.007	---	0.020	0.021	n.i.
19.89	---	---	---	---	---	✓	✓	✓	✓	n.i
20.12	0.013	0.017	0.014	0.012	0.016	0.016	0.024	✓	0.009	Syringyl -CH=CH-CHO (Sinapaldehyde)
20.27	---	---	✓	---	✓	---	---	---	✓	Palmitic acid
20.35	0.014	0.018	0.011	0.009	0.010	0.009	✓	✓	✓	n.i.
20.56	0.122	0.119	0.172	0.138	0.114	0.105	0.018	0.139	0.174	n.i.

To be continued ↓

Ret.time	Proportion of the substances [%]									Substances
	db					wb				
	Beech	Ash	Oak	Chest	Mix	Beech	Ash	Oak	Chest	
21.04	0.009	0.023	✓	✓	✓	0.010	0.015	0.009	✓	n.i.
21.20	✓	✓	✓	✓	✓	✓	---	---	✓	Syringyl -CO-CH_2-CH_2OH
21.24	---	---	---	---	---	---	0.017	---	---	Isofraxidin or derivative
21.47	---	---	---	---	---	---	0.072	---	---	Fraxinol or derivative
21.55	✓	✓	✓	✓	✓	✓	✓	0.009	✓	n.i.
22.02	---	---	---	---	---	---	---	---	---	Stearic acid
24.40	---	---	---	---	---	---	---	---	---	Internal Standard
26.50	✓	0.013	✓	✓	✓	0.012	0.011	✓	✓	n.i.
27.58	---	---	0.029	✓	✓	✓	---	0.038	0.007	n.i.
31.44	0.028	0.050	0.043	0.040	0.046	0.043	0.070	0.022	0.031	Lignan (Syringaresinol)
SUM	0.620	0.820	0.789	0.673	0.577	0.602	0.816	0.698	0.746	

✓ = < 0.005 mg/ml ≥ 0.001 mg/ml --- = Peaks not properly detected n.i. = not clearly identified

Table presents the proportion of the substances based on the solid matter of the LPS

5.9 Mineral components in wood, SPS and LPS

On the first sight, mineral constituents just account to a very small proportion of the raw material and thus seem to be less important for a biorefinery process, which targets the utilization of the individual organic wood components. However, on the second sight, it gets clear that this fraction accumulates somewhere in the machinery or process streams as mineral components themselves are not converted during the process. With this, it becomes interesting to follow the accumulation or leaching of minerals from the different process streams to evaluate potential adaptations of the biological conversion step or the waste stream management. Therefore, this chapter presents the summarized results about the mineral components. This enables a direct comparison and tracking of the leaching and distribution of the mineral components over the process. This included the analysis of the raw materials as well as the SPS and the LPS. These analyses were mainnly performed with the materials with one-origin materials, and thus partially excluded the mixed sample.

5.9.1 Quantification of mineral components in wood and the SPS

The leachability of mineral components is dependent on the type of minerals and their individual solubility. Further, it depends whether the minerals are involved in physiological processes in the sapwood, are bound within the cell walls or originate from impurities like soil particles sticking to the tree stems (Hörhammer et al., 2018; Kenney et al., 2013; Reza et al., 2015).

For combustion processes the negative effects like slagging or equipment fouling are known, which are initialized by minerals. However, some studies also found that a reduction of the ash content prior to processing had positive effects on bioconversion processes like enzymatic hydrolysis and fermentation (Bin & Hongzhang, 2010; He et al., 2014). Hörhammer et al. (2018) found an increased monomeric sugar yield for short rotation poplar, washed under different condition prior to steam explosion and enzymatic hydrolysis or fermentation. However, it could not be clearly shown if this effect was solely due to the decrease of mineral contents or also through extraction of inhibitory substances like sugar degradation products.

In this thesis the first step to track the mineral components was to determine the ash content of wood and the SPS. Additionally, the ash determination was done for hot water extracted wood. This step was added to compare the leaching after steam explosion under acidic conditions with the leaching effect under natural pH conditions.

The results are presented in Figure 15 (a - c). In graph **a) the mineral content for wood** is visualized. For the **debarked samples** the mineral content was found to be between 0.11 - 0.56 %, with lowest values for oak (0.23 %) and chestnut (0.11 %).

Fengel and Wegener (2003) specified a mineral content of 0.2 - 0.5 % as the typical range in wood of the temperate zone. This range fitted for the values of the debarked samples determined here. The slightly lower and higher values could be due to the variation of environmental conditions or different analytical procedures, for instance different incineration temperatures (Fengel & Wegener, 2003). The low mineral content determined for chestnut potentially supported the theory of the young age of the harvested trees, since the period in which the tree could take up and store minerals was shorter than for the other tree species. This was further supported by the corresponding low amount of minerals for chestnut with bark, which could be seen as an indication for a low bark proportion.

Wagenführ and Scheiber (1974) displayed some values for the mineral content of different tree species, with 0.3 - 0.6 % for *Q. robur/petrea.*, 0.4 - 0.6 % for *F. excelsior* and 0.3 - 1.2 % for *F. sylvatica* as well as 0.3 - 0.4 % for *C. sativa*. Comparing these values with the results of this thesis, the values showed a good agreement for beech and ash, whereas the values for oak

and chestnut were a bit low, but these variations were still explainable by natural variability and difference in age.

For **samples with bark** the detected mineral content was higher 0.63 - 1.61 %, with highest values for ash wb. The mineral content in bark was reported to be 10 times higher than for wood with Ca as main element, followed by K and Mg. These elements were also dominant in wood with Ca in wood accounting to 50 %, but in bark to 82 - 95 % (Fengel & Wegener, 2003).

For the pure bark of *F. sylvatica*, a mineral content of 7.3 % and for *Q. robur* of 2.2 % was detected (Dietrichs et al., 1978), which is much higher than the determined values. Zając et al. (2018) included “wood residue chips” originated from forests or municipals into their study about chemical characteristics in biomass ashes. For these samples, mineral contents of 0.96 % (forest) and 1.79 % (municipals) were found. These samples likely contained bark proportions as these were described as residues and were separated from wood samples, but there is no further specification included in the study. Therefore, the values determined in this thesis for samples with bark showed good agreement to the values from literature as the samples analyzed here were wood chips of not-debarked stems, leading to a mixture with high wood proportion > 80 %.

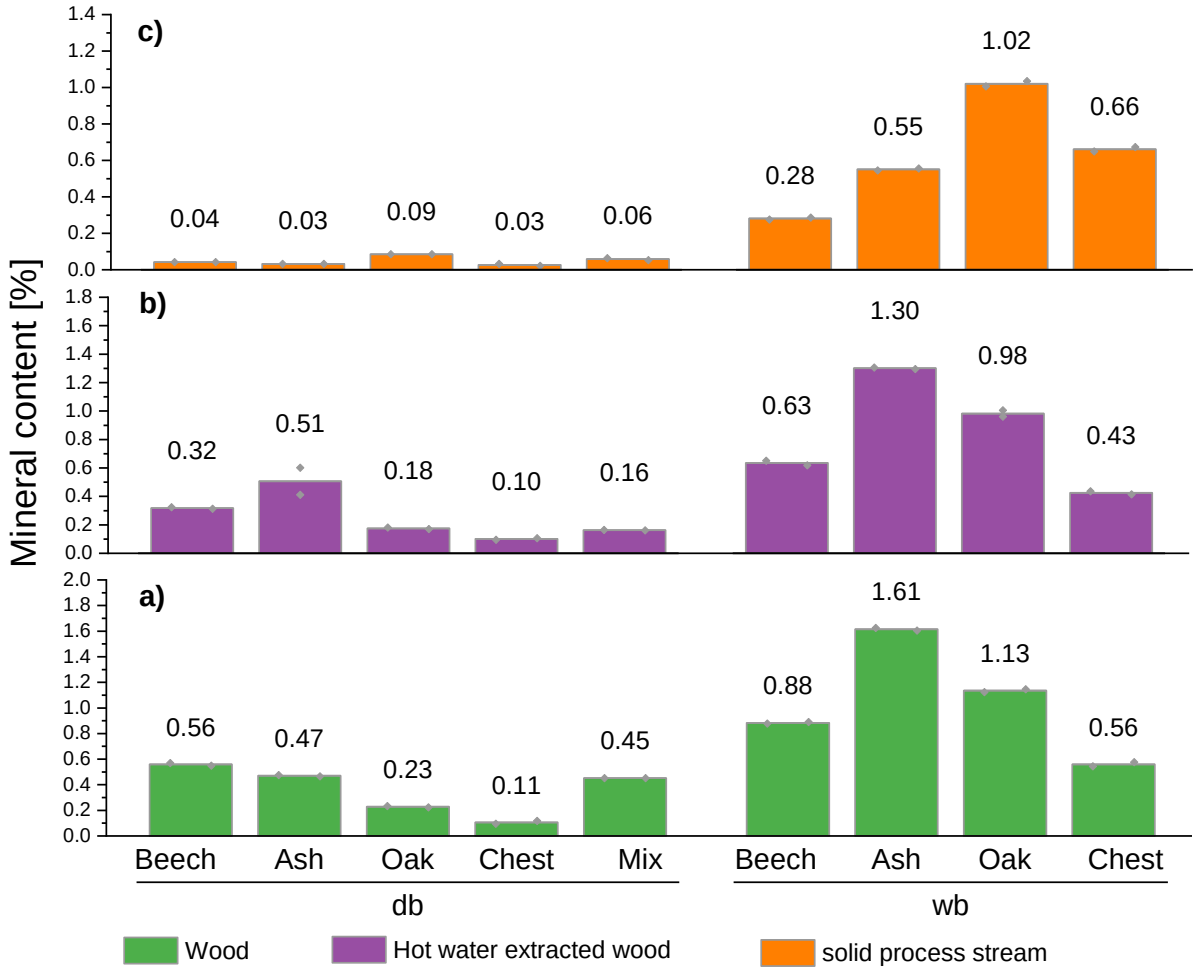


Figure 15: Mineral content after incineration at 525 °C for wood a), hot water extracted wood b) and the SPS c); the bars and values represent the average values and the symbols the individual measurements.

In Figure 15 b) **the mineral content of hot water extracted wood** is shown. This enabled to study the leaching effect under natural pH conditions, solely dependent on the trees pH in aqueous medium. For the **samples debarked** changes in the mineral content ranged from +9 % for ash db to -64 % for the mixed sample. The detected increase is likely a relative effect, due to the removal of the hot water soluble substances. For the **samples with bark** the decrease of the mineral content ranged between -13 % to -28 %. This indicated that the leaching effect differs between the species, but the ratio grading between the mineral content of the species stayed the same, except for ash db. The observed variations between the species were probably due to different mineral compositions, environmental conditions as well as the binding form and location within the cell structure and resulting accessibility and solubility during hot water extraction (Mai & Zhang, 2023; Werkelin et al., 2010).

Comparing these results with the **mineral content** determined **for the SPS** in Figure 15 **c)** the leaching effect was intensified in most cases, especially for the debarked samples. The **db samples** showed residual mineral contents of 0.03 - 0.09 % and interestingly ash db now had the lowest mineral content. The **samples with bark** showed residual mineral contents of 0.28 – 1.02 %. However, for oak wb solely a very low decrease in the mineral content was detected and for chestnut even an increased mineral proportion was found, if compared to the mineral contents in wood. Additionally, a shift in the grading between the tree species was observed, as now oak wb and db showed the highest mineral content instead of ash.

The observed effects could possibly be explained by the acidic conditions during the pretreatment in combination with the effect of steam explosion itself. The steam explosion changed the accessibility of the material, as the cell structure is destroyed, which potentially increased the availability of mineral compounds normally integrated in the cell complexes. Additionally, the acidic conditions were preferable for the dissolution of Ca and Mg carbonates or oxalates (Hörhammer et al., 2018). Hörhammer et al. (2018) also detected a decrease of the mineral content about 80 % for acidic washing and 59 % for acidic followed by neutral washing. In contrast, solely 26 % of the minerals were reduced during washing under neutral conditions (Hörhammer et al., 2018). Several other authors also found a positive effect of acidic pretreatment by a higher removal of mineral compounds (Aston et al., 2016; Chin et al., 2015).

Regarding the results for oak and chestnut with bark the acidic pretreatment and destruction of the cell wall structure seemed to have less effect than for other species and also compared to the condition during hot water extraction. For chestnut wb, even a slight accumulation of minerals within the SPS was observed. One explanation could be that this is a relative enrichment due to the removal of other wood components like hemicelluloses during the pretreatment. If this effect is paired with the high mineral content of the samples with bark and a probably lower leachability of the minerals in this species potentially led to the observed values, as these effects overcompensated the actual leaching. Werkelin et al. (2010) for instance observed a good leachability of the Ca fraction during acidic conditions, but the proportions containing S and Si stayed insoluble. Especially, the proportion of Si could be increased in the samples with bark due to the presence of externally introduced ashes originated from attached soil particles (Brown, 2019).

Overall, especially for the debarked samples a strong leaching was detected due to the SE pretreatment with a reduction up to -94 %. This indicated that the leached minerals have to be found in process streams other than the SPS.

5.9.2 Screening the elemental composition

To get a first overview on the elemental composition of the mineral residues after incineration EDX analyses were performed. EDX analyses evaluate the presence of an element, by peak intensity, but in ratio to all elements in the sample. Therefore, this technique allows no absolute quantification, but the evaluation of changes in elemental ratios, which indicate potential leaching effects during the processing of the material.

Wood

The EDX analyses of the mineral residues of wood showed K and Ca as main elements with highest peak intensities. For beech, ash db/wb, the mixed sample as well as oak db the K peak outraged the Ca peak, but for oak wb and chestnut db/wb it was the other way around. This potentially indicated different elemental proportion in these species (Figure 16). Other elements detected by EDXA were Mg, Na, Si, P and S, but also some oxygen (O). The latter one could be present as some oxides get normally formed during incineration. Another reason could be that the incineration at 525 °C was probably too low in temperature to completely remove all organic elements or led to an insufficient removal of alkaline earth metal carbonates by oxidation. However, it ensured the preservation of the complete element profile by preventing for example losses of alkali metal chlorides (Fengel & Wegener, 2003). As the determined mineral contents in the prior chapter agreed with the ranges given by literature, the method seemed to be suited for the question addressed here.

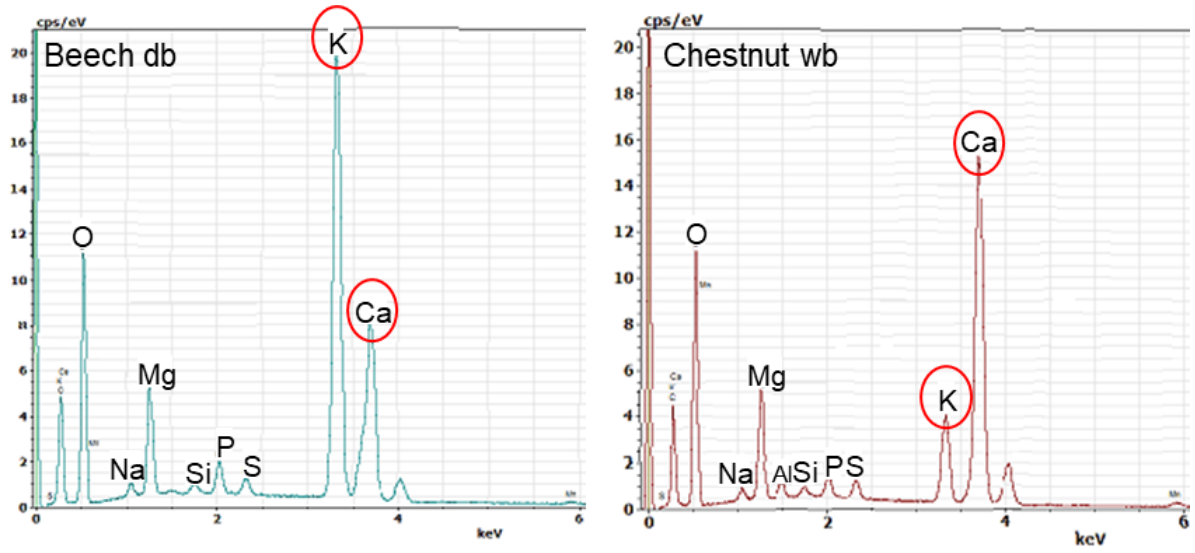


Figure 16: Spectra of EDX analyses exemplary for the mineral residue after incineration of beech db (left) and chestnut wb (right) wood.

Hot water extracted wood

For the hot water extracted samples, a strong decrease of K in ratio to Ca was detected for all species db and wb. This is due to the good leachability of potassium related compounds like KCl, K_2HPO_4 and K_2SO_4 under neutral conditions (Hörhammer et al., 2018). The elemental shift is visible by comparison of Figure 16 with Figure 17.

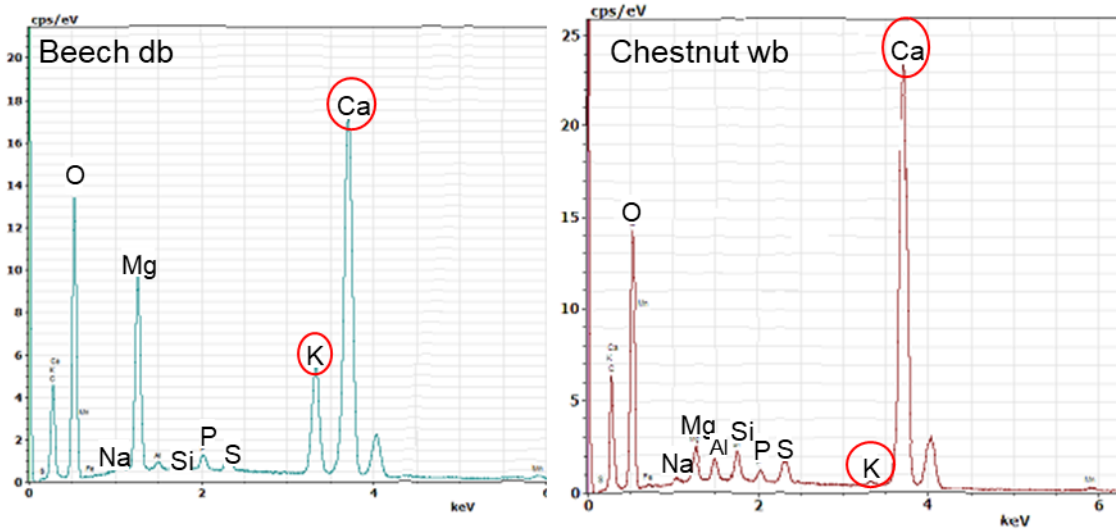


Figure 17: Spectra of EDX analyses exemplary for the mineral residue after incineration of hot water extracted wood of beech db (left) and chestnut wb (right).

Solid process stream (SPS)

The leaching effect seemed to be even more pronounced for the mineral content of the SPS (see Figure 18). Especially, for the debarked samples, the proportion of calcium was observed to decrease as well as the potassium proportion. This was assumed because the peak intensity ratio towards the other elements like Si, Na and P was decreased. These observations agreed with the detected strong reduction of the total mineral content in the SPS, described in chapter 5.9.1. The strong reduction of calcium in the SPS was likely caused by the acidic conditions during the material processing. At lower pH different minerals like carbonates and oxalates, which are mainly aligned to Ca or Mg atoms become soluble and thus leachable (Hörhammer et al., 2018). Additionally, Werkelin et al. (2010) also observed an increased Ca leaching at acidic conditions, but mainly within bark samples.

In relation to the EDX analyses, the effect seemed to be less strong for samples including bark, as the Ca peak is still very present. However, this could also be traced back to the variations of the elemental frequency in bark with a higher Ca and a lower Mg and Mn content compared to wood, resulting in a still high proportion of the Ca despite leaching (Fengel & Wegener, 2003).

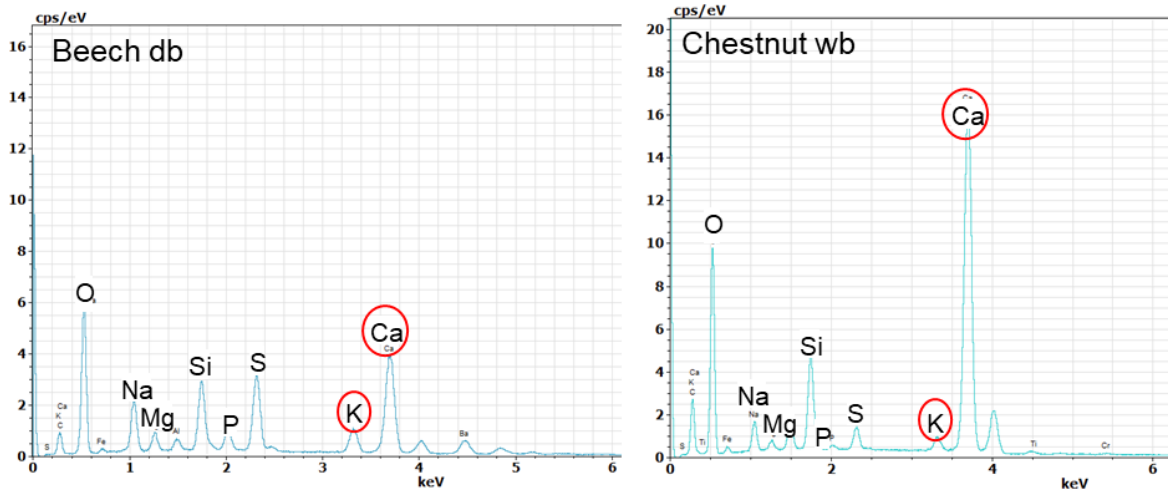


Figure 18: Spectra of EDX analyses exemplary for the mineral residue after incineration of the SPS of beech db (left) and chestnut wb (right).

5.9.3 Quantification of the individual elements

The EDX analyses showed some shifts in the frequency of individual elements, but analyses were solely possible for the mineral residues after incineration, excluding the LPS. Further, no absolute quantification of the elements was possible and thus ICP analyses were performed as well. These analyses were applied on the original materials wood, SPS and LPS, without prior incineration of the material. The presentation of the sulfur (S) content was solely presented for the raw material wood, as for the SPS and the LPS the values were probably distorted by the pretreatment.

Wood

The results of the ICP analyses of wood are presented in Table 19. For the **debarked samples** the main elements were Ca, K and Mg. For beech, ash and oak **K** was the element with the highest abundance of 452 – 1220 mg/kg. Interestingly, for chestnut no K was detected. This result was unexpected, as the element was detected in the EDX analyses and K was described as a macronutrient, which is important for the turgor regulation within the cells (Matyssek et al., 2010). It is likely, that K is present in chestnut db, but in low quantities and thus below the detection level of the method, which was given with 250 mg/kg. Following this argumentation, this is a further indices for the young age of the chestnut trees, as the time period for the accumulation of metals was much shorter than for the other trees (Zając et al., 2018).

The values for **Ca** with > 700 mg/kg detected for beech and ash db were found to be nearly double the amounts detected for oak and chestnut db. If that comparison is transferred to the samples with bark, the general higher Ca content described for bark in literature (Fengel & Wegener, 2003) became obvious. Additionally, a correlation between the bark proportion described in chapter 5.1 and the Ca content of the respective tree species was indicated. This was because ash and oak were the species with the highest bark proportion and in agreement with that showed very high Ca contents of 4120 mg/kg (ash wb) and 3250 mg/kg (oak wb) for calcium. However, the difference between db and wb samples was strongest for oak, where the Ca content increased about a factor of eight.

Zając et al. (2018)[1] detected round about 580 mg/kg calcium for oak and 1203 mg/kg for ash, which was a bit higher than the here detected values for debarked samples. However, for pure ash and oak bark values of 28465 mg/kg and 20220 mg/kg were presented, which was much higher than in the samples with bark analyzed in this thesis. This was to be expected as Zajac et al. (2018) analyzed pure bark and not a mixture of wood and bark with a wood proportion of > 80 %. Under the assumption that the described “wood residue chips” originated from forests or municipals in the study of Zając et al. (2018)[1] included wood and bark proportions the respective Ca proportions were roughly 1958 mg/kg for wood residues from forests or 4387 mg/kg if originated from municipals. These values were in agreement with the values for samples with bark, presented here.

The effect of bark was additionally obvious in the case of the element **Si**, especially for beech and ash. The content of the db samples was < 360 mg/kg, but samples with bark showed proportions of >1000 mg/kg and > 2500 mg/kg, which was likely influenced by proportions of externally introduced minerals from soil particles. Interestingly, the opposite effect was detected for oak, where the wb samples contained less Si than the db samples. This could be explained by the possible high variations regarding the amount of externally introduced Si.

[1]Values from Zajac et al. (2018) were recalculated manually to be based on the raw material

The element **S** was described as a macro nutrient in wood (Mai & Zhang, 2023), however it was solely detected in ash and oak for the debarked samples with values over the detection limit of 125 mg/kg. For the samples with bark, sulfur was detected in all species, except of chestnut with bark. Interestingly, the presence of bark seemed to enhance the content of S similar to Ca, but in a lower extent. Zając et al. (2018)[1] detected 19 mg/kg S in oak wood and 13 mg/kg in ash wood, which represented lower values than the here detected contents of 131 mg/kg for oak db and 142 mg/kg for ash db. The reported values of the pure bark samples of oak and ash with 602 mg/kg and 305 mg/kg in contrast were much higher than the here observed values for samples with bark, which ranged between 114 – 260 mg/kg. Additionally, also the values for the forest residues, which probably included bark showed lower values than the here detected contents for samples with bark, at least compared to ash and oak db.

Regarding the elemental composition, especially oak wb is interesting as it was observed to contain 265 mg/kg of **P**, which is an important macro nutrient for the trees metabolism. Additionally, oak wb contained 177 mg/kg **Na**, which is not an essential nutrient element, but seems to be still accumulated (Matyssek et al., 2010). Furthermore, chestnut db is the only sample where **Cl** was measured in a volume above the detection limit. These elements (Na, Cl and P) were not detected for the other species. As Na and P were detected during the EDX analyses it was likely, that these elements were present in wood, but in amounts below the detection limits of 125 mg/kg. The detection limit for Cl was 50 mg/kg. Some additional ICP analyses of the mineral residues after incineration of ash db and wb, revealed detectable amounts of P, Na and also Cl (data not shown). This clearly supported the assumption, that the elements were present, but the amounts were too low to be detected in wood.

The same context could explain the absence of the other known micro nutrients like Cu or Mo, but also for example for the heavy metal Hg. This element was only detected in wood with bark of chestnut with 1.56 mg/kg and a detection limit of 0.46 mg/kg.

Another element, which was detected in the samples was **barium (Ba)**, despite the fact, that this element is not known to contribute to the trees metabolism. However, it was reported in literature, that Ba can be taken up by plants, most easily from acidic soils. The availability of Ba for an uptake depends on the soil composition, next to other factors. Ba contents of 0.5 – 40 mg/kg were reported for plants on normal soils (Madejón, 2013).

[1]Values from Zajac et al. (2018) were recalculated manually to be based on the raw material

Table 19: ICP results of the wood samples; values are given as mg per kg dry wood

		Wood							
Element	**DL**	**Beech**	**Ash**	**Oak**	**Chestnut**	**Beech**	**Ash**	**Oak**	**Chestnut**
		db				**wb**			
	[mg/kg]	**[mg/kg]**							
Cl	50.00	---	---	---	461.5	---	---	---	---
Na	125.00	---	---	---	---	---	---	176.88	---
K	250.00	1220.00	1060.00	452.10	---	1050.00	2160.00	815.15	---
Ca	12.50	737.16	768.80	385.62	329.04	1940.22	4120.00	3250.00	1900.00
Mg	1.25	218.06	191.91	35.15	44.23	340.82	344.07	163.69	184.56
Fe	12.50	---	26.59	---	---	---	---	13.21	---
Al	12.50	16.22	20.65	22.51	15.45	19.28	22.86	31.50	31.70
Si	12.50	317.04	359.72	972.34	85.36	1040.00	2560.00	217.66	920.89
S	125.00	---	142.52	130.62	---	113.92	259.78	240.23	---
P	125.00	---	---	---	---	---	---	264.47	---
Ba	2.11	10.35	3.22	6.84	4.08	32.01	15.53	12.50	16.83
Sr	0.65	3.44	3.56	1.76	2.01	5.75	11.82	7.13	6.75
Mn	1.25	22.79	---	11.97	5.57	71.76	13.78	19.01	44.50
Ti	12.50	---	---	---	---	---	---	---	---
Cu	2.21	---	---	---	---	---	---	3.96	---
Hg	0.46	---	---	---	---	---	---	---	1.56
Mo	0.40	---	---	---	---	---	---	---	---
W	1.17	---	---	---	---	---	---	---	---

DL = detection limit db = debarked wb = with bark --- = not detected

Solid process stream (SPS)

Switching to the results for the SPS, presented in Table 20, the elements **Na, K** and **P** were not detected for any species db/wb. These results strongly indicated a heavy leaching, which supported the findings of the previous analyses, especially for K. Further, the micro nutrient **Mn** was not detected anymore except for ash wb. Interestingly, the wood sample of ash wb, was not the one with the highest initial Mn content. This effect could be probably explained by a relative effect or the Mn was allocated with other elements, which influenced the leachability. Hörhammer et al. (2018) for instance found no leaching of Na in steam exploded poplar after acidic preprocessing, but P, K, Mg and Mn were removed completely.

The comparison of the mineral content between wood and the SPS is based on the amount of the respective elements per kg of wood or SPS. For the interpretation it has to be considered, that one kg wood did not result in one kg SPS and the conversion factor is not known. This means that an accumulation has to be understood as a relative enrichment.

Comparing the contents of **Ca** between wood and SPS it was found that the contents strongly decreased, especially for the debarked samples. Around 7 - 17 % Ca remained in the SPS, except for oak db, where a Ca content of ~ 60 % remained. This effect was even stronger for the samples with bark. There was a strong decrease in the Ca content for all species (wb), but for oak wb even a small allocation was detectable as the content was ~ 106 % compared to the initial content in wood. Next to oak wb, also for chestnut wb ~ 77 % of the initial Ca content remained in the SPS.

In contrast, the amounts of **Mg** were strongly decreased for all samples. The highest remaining proportion were detected for oak db, but no special effect for the wb samples, like for Ca, was detected for Mg. The element **Cl,** which was already detected in wood was still present in the SPS of chestnut db, with a slight increase. Additionally, Cl was detected in the SPS of ash db, but in a low proportion. In literature, it was reported that Cl was easily leached with water and that Cl was mainly present as KCl salts, which are easily dissolved by water (Werkelin et al., 2010).

Regarding to the micro nutrients, **Fe** seemed to be not leached from the SPS of the samples with bark or even an accumulation was detected. This assumption was based on the observation that Fe could not be detected in wood, but was detected in the SPS for the samples with bark and oak and chestnut debarked. It was assumed that this was again an effect of component ratios. Therefore, the missing Fe proportion in the SPS of beech and ash db could be explained by the option that the removal of other components during the SE process could simply not increase the proportion of Fe above the detection limit. These findings do not correlate perfectly with literature, which described a decrease of the Fe and Al proportions during acidic washing, at least for some proportions (Werkelin et al., 2010).

However, it also had to be considered, that probably some mineral proportions originate from the machinery, for example due to corrosion.

Comparing the **Si** content of wood with the respective SPS showed a strong reduction for all species db/wb, except for ash db, where a slight accumulation was detected. The mainly observed reduction of the Si content is probably due to the proportions of externally introduced minerals, as these are easily removed by washing. However, it was also reported that non-metal Si in woody biomass was mostly insoluble (Werkelin et al., 2010), which could explain the remaining proportion in the SPS.

The element **Ba** also seemed to be partly non-soluble as an increased Ba content in the SPS compared to the wood could be observed for beech db as well as oak and ash wb. One possible explanation could be the difference in solubility of Ba salts as $BaCO_3$ and $BaSO_4$ were

described with a low solubility, but barium chlorides and nitrates were described to be better soluble (Madejón, 2013).

Hg as heavy metal was solely detected in the wood of chestnut wb, but was observed in nearly all samples of the SPS, especially samples with bark in a ratio of ~1 mg/kg. Plants can take up Hg in low proportion from soils, but also from the atmosphere. However Hg forms strong bonds to organic matter in soils (Steinnes, 2013), thus probably also with the organic wood proportions after uptake, which would explain the accumulation due to ratio changes.

Table 20: ICP results of the SPS samples; values are given as mg per kg dry SPS

Solid process stream

Element	DL	Beech	Ash	Oak	Chestnut	Beech	Ash	Oak	Chestnut
		db				wb			
	[mg/kg]	[mg/kg]							
Cl	50.00	---	49.80	---	513.20	---	---	---	---
Na	125.00	---	---	---	---	---	---	---	---
K	250.00	---	---	---	---	---	---	---	---
Ca	12.50	71.37	53.38	230.02	58.26	922.18	1700.00	3430.00	1460.00
Mg	1.25	6.90	7.93	16.57	9.16	11.67	22.06	10.06	16.23
Fe	12.50	---	---	24.23	19.88	23.21	90.73	35.44	59.50
Al	12.50	---	12.51	18.93	20.28	37.62	55.02	106.28	73.23
Si	12.50	71.22	468.79	59.10	77.20	130.25	56.80	59.22	69.34
P	125.00	---	---	---	---	---	---	---	---
Ba	2.11	25.09	---	6.90	---	28.22	36.18	10.85	38.19
Sr	0.65	1.65	---	---	---	2.64	3.16	6.11	5.60
Mn	1.25	---	---	---	---	---	3.34	---	---
Ti	12.50	---	---	---	---	---	---	---	22.11
Cu	2.21	---	---	2.48	---	---	---	2.27	---
Hg	0.46	1.18	---	1.31	---	1.19	1.79	0.77	0.58
Mo	0.40	---	0.38	0.53	0.53	0.54	0.51	0.66	1.51
W	1.17	---	---	---	---	---	---	---	1.32

DL = detection limit db = debarked wb = with bark --- = not detected

Liquid process stream (LPS)

The interpretation of the elemental composition of the LPS was difficult. This was because the ICP analysis was performed directly on the liquid and as already discussed in prior chapters the substance concentration could not be taken as a constant variable as it was dependent on the process and sample taking procedure. Therefore, the data in Table 21 presents the data converted to mg per kg of dried LPS [mg/kg] applying the determined values for the solid matter (see Table 10). Due to the conversion of the values, the detection limits were not included in Table 21. The mineral proportions of the LPS clearly showed that the leached elements from the SPS were at least partly included in the LPS. It had to be considered, that there were potentially additional process streams included in the SE pretreatment, which were not comprised in the frame of this thesis.

The element **Ca** showed high values between 440 mg/kg – 1158 mg/kg. Interestingly, the lowest Ca values belonged to the LPS of ash debarked and the highest values to the LPS of ash with bark. This clearly supports the assumption of Ca leaching under acidic conditions as discussed for the SPS and was for example shown by Werkelin et al. (2010). **K** and **Mg** were also notable present in the LPS with proportion for K of 59 mg/kg for chestnut db and up to 1160 mg/kg for ash wb. Regarding the Mg proportion, the range was 30 mg/kg for oak db up to 163 mg/kg for ash wb. Like K, the **Na** was not detected in the SPS, but was measured in the LPS with 84 mg/kg – 237 mg/kg. Additionally, **P** and micro nutrients like **Mn, Co, Cu, Zn** were present in in the LPS, which were not observed in the SPS and the wood. This clearly proved the presence of these elements in wood and showed the leachability.

The observed accumulation of **Ba** and **Mo** (not discussed) or **Hg** in the SPS were supported by the analyses of the LPS as these elements were not detected or just in very low proportions, which showed a very weak leaching of these elements. The proposed strong leaching effect of **Si** during the conversion of wood into the SPS, could not be supported by the detected proportions of Si in the LPS, as these seemed quite low compared to the discrepancy between wood and the SPS. This led to the assumption that the leached Si proportion potentially accumulated in a different process stream. For example, if there would be a simple washing step of the wood chips prior to the pretreatment, the externally introduced Si proportion would be already removed.

Within the debarked samples, the LPS of beech seemed to carry the highest mineral load, with just some small exceptions. Oak and chestnut db for instance carried the highest volumes of **Ni** with > 2 mg/kg and also of **Cr** with values > 3 mg/kg. Comparing the debarked samples with the samples including bark the mineral proportions were almost always notable higher, which correlated with the knowledge about the higher mineral content in barks compared to wood (Fengel & Wegener, 2003) This effect was also present for the wood and the SPS. Ni is known to be a micro nutrient in plants (Matyssek et al., 2010), but Ni as well as Cr potentially also could be originated from the materials used for the machinery. Additionally, also arsenic (As) was observed in the LPS. This element has no function in the trees metabolism, but it can be taken up from soils by plants (Wenzel, 2013).

The here determined values for the LPS lack values from the literature for comparison, as studies about leaching mainly focus on the solid process stream and the improvements of following conversion processes due to leaching (Aston et al., 2016; He et al., 2014; Hörhammer et al., 2018).

Overall, the analyses of the mineral components within wood the SPS and LPS showed the different distribution patterns and some differences between the species. These analyses mainly contributed to 1.2 and 1.3 as well as 2.2.

Table 21: ICP results of the LPS samples converted to mg per kg dried LPS [mg/kg]

Element	Liquid process stream (LPS)							
	Beech	Ash	Oak	Chestnut	Beech	Ash	Oak	Chestnut
	db				wb			
	[mg/kg]							
Na	123.29	117.47	84.49	103.77	119.70	237.31	132.42	103.31
K	357.17	377.48	266.64	59.30	376.62	1159.76	484.17	106.14
Ca	625.23	439.92	538.60	410.80	776.55	1158.45	894.12	974.21
Mg	123.97	96.45	29.59	41.99	121.61	163.42	94.01	80.23
Fe	14.34	13.18	13.22	12.77	25.37	10.22	12.74	40.03
Al	23.42	15.54	12.64	12.86	23.11	17.61	16.23	28.20
Si	45.39	26.58	43.15	34.22	85.24	43.49	58.03	54.76
P	166.28	132.64	138.71	49.39	146.73	178.62	444.27	113.60
Ba	0.45	0.16	0.28	0.29	0.19	0.65	0.64	0.62
Sr	3.45	2.54	1.72	2.18	2.25	6.87	3.08	3.80
Mn	38.56	0.98	6.40	3.49	25.41	7.32	12.32	15.66
Cr	1.04	0.86	3.11	3.53	2.03	1.37	1.15	3.16
V	0.02	0.01	---	---	0.04	0.02	---	---
Zn	2.13	1.89	2.04	1.04	2.67	4.90	6.86	7.77
As	0.01	0.01	0.03	0.02	0.02	0.02	0.04	0.02
Cd	0.04	---	0.03	0.01	0.02	0.01	0.05	0.01
Co	0.21	0.55	0.16	0.11	0.24	0.41	0.10	0.08
Cu	1.11	1.23	1.59	0.92	2.25	2.40	0.93	0.97
Hg	---	---	---	---	---	0.04	---	---
Mo	0.01	---	---	0.01	---	---	0.01	0.01
Ni	0.99	1.17	2.58	2.41	1.10	1.52	1.16	2.05
Pb	0.06	0.07	0.10	0.06	0.05	0.09	0.16	0.08
Sb	0.00	0.00	---	---	---	0.00	0.00	0.00
Tl	---	---	0.01	---	---	---	0.01	0.01
W	0.07	0.04	0.06	0.08	0.18	0.10	0.07	0.06

db = debarked wb = with bark

5.10 Proportionate composition of the different process streams

One target of a lignocelluloses based biorefinery is to utilize the raw material completely (BÖR, 2023). To be able to apply this approach it is necessary to understand the different proportions of common wood components and the variation between the species. This enables to choose suitable species in regard to the aimed products and component proportion within the raw material. Further, it points out potentials for new or additional utilization routes. Therefore, it was decided to include a short summary of the here determined proportions of the different materials. These results thus contribute to research question 1.3.

Wood

For wood as raw material, Figure 19 presents the mass balance for each species to summarize the raw material characterization at least from a quantitative perspective. Based on the frame of this thesis the main target product was glucose and thus species with a high proportion of glucan would be favorable. Regarding Figure 19, the strongest species-specific variations were detected for the proportions of glucan and the extractives.

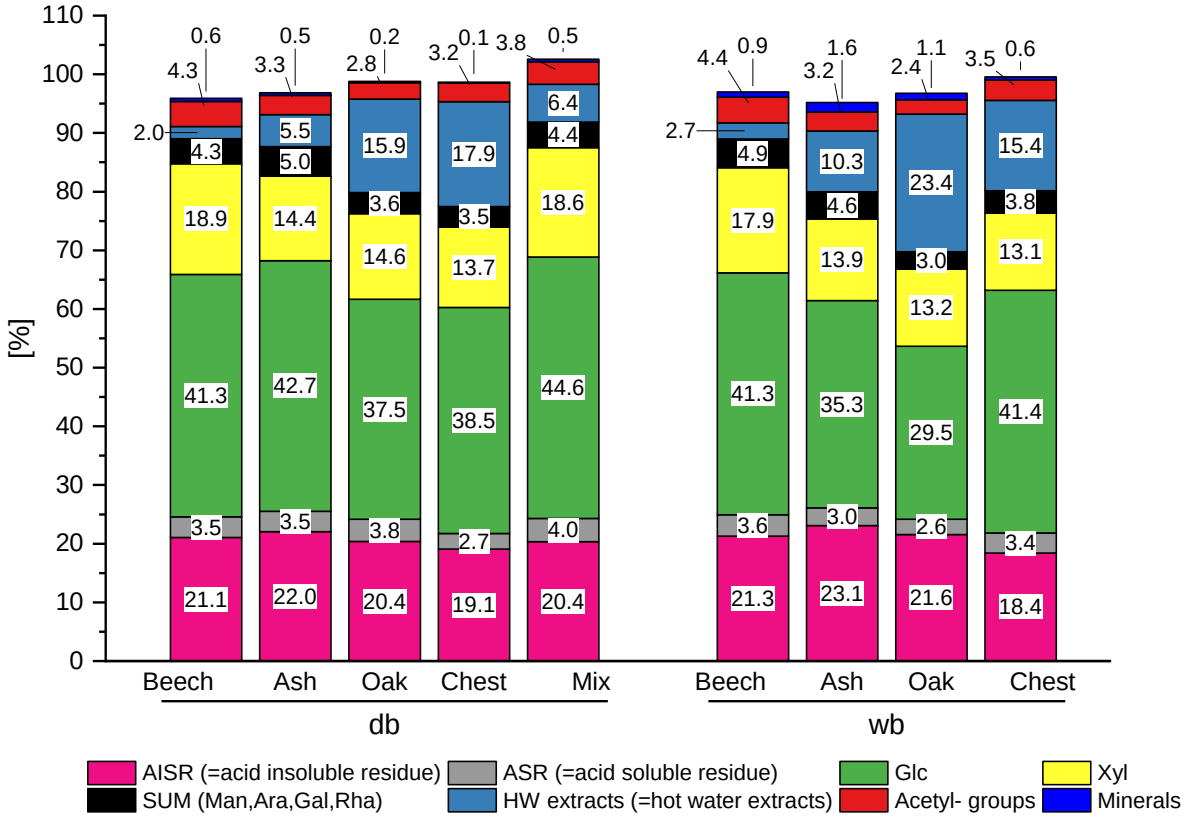


Figure 19: Mass balance of wood according to the performed analyses; for the mineral content the values from the incineration experiment (see chapter 5.9.1) were used.

For beech debarked the ratio of the glucan proportion to the proportion of the hot water extracts was found to be 20.5. In contrast, for oak and chestnut debarked this ratio was 2.3 and 2.2. For the samples with bark, the influence of bark was also strongly visible, especially, for oak, were the ratio decreased to 1.3. Therefore, the ratio of convertible material per log would be more preferable for beech than for oak or chestnut. At the same time, the big proportions of extractives points out the potential to use this fraction for the production of further valuable

products, next to glucose. Regarding the aim to receive a mass balance of 100 %, Figure 19 showed a good fit for oak db and chestnut db as well as wb. However especially for beech and ash some proportions were missing, potentially assigned to pectins or uronic acids, which were not included in the analyses. Potential reasons for recoveries > 100 % (mixed sample) were already discussed in chapter 5.4.

Solid process stream (SPS)

Regarding the mass proportions the SPS could be characterized by the proportions of sugar and lignin or lignin-like substances determined during acid hydrolysis (see chapter 5.4). Further, the mineral content was included, determined by incineration (compare chapter 5.9.1).

Overall, this summation of the detected proportions showed that the SPS seemed to mainly contain sugars, next to AISR and ASR as well as a very small proportion of minerals. Potential reasons for recoveries ≥ 100 % were already discussed in chapter 5.4. Further, missing proportions could be due to degradation products or extractives, which were dissolved during acid hydrolysis, but not quantified by UV measurements.

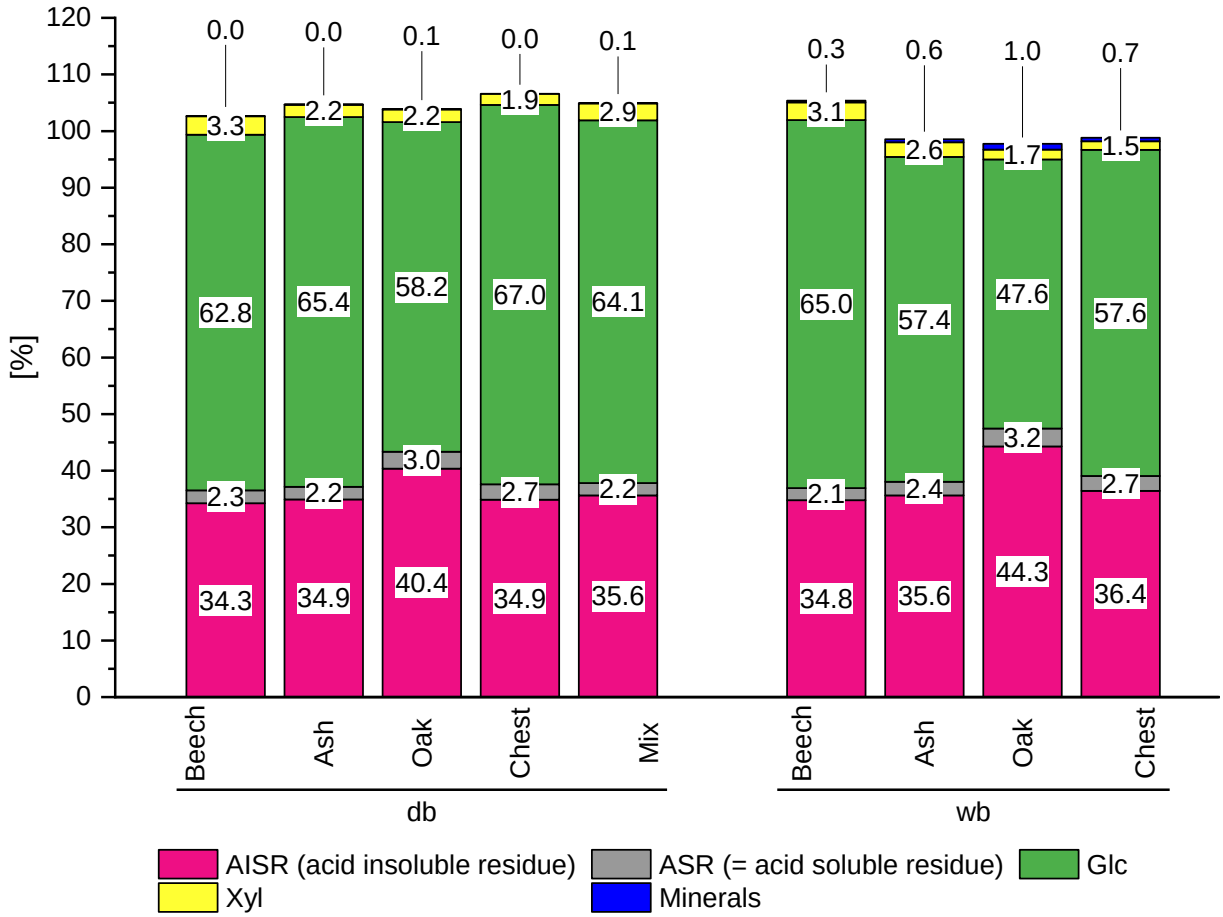


Figure 20: Mass proportions of the SPS.

Liquid process stream (LPS)

In Figure 21 the proportions of the different fractions are presented, based on the dry LPS. The sugars clearly accounted for the major proportion with > 57 % of the LPS solid matter. The second largest proportion was represented by the sugar degradation products with proportions ≥ 8 %. This showed that 70 - 80 % of the LPS (solid matter) were sugars or their degradation products. The residual 20 - 30 %, thus have to account to other degradation products or extractives and minerals. By solid phase extraction (SPE) between 5.7 – 14.2 % of the LPS were extracted. That meant for instance that 92.5 % of the dry LPS of chestnut wb was represented by sugars, sugar degradation products and the extracted fraction. The proportion of the DCM extraction was not included here, as the extract was shown to contain partly similar components than the SPE extract and some sugar degradation products. For reference, a detailed description of these fractions were already presented in chapter 5.8.2. The mineral proportion was the smallest fraction with values between 0.3 - 0.6 %. Interestingly, ash and oak wb contain the highest mineral proportions, which correlated with the high bark proportions compared to beech and chestnut. For the mixed sample, no value for the mineral content was included as this sample was not included in the ICP measurements of the LPS samples.

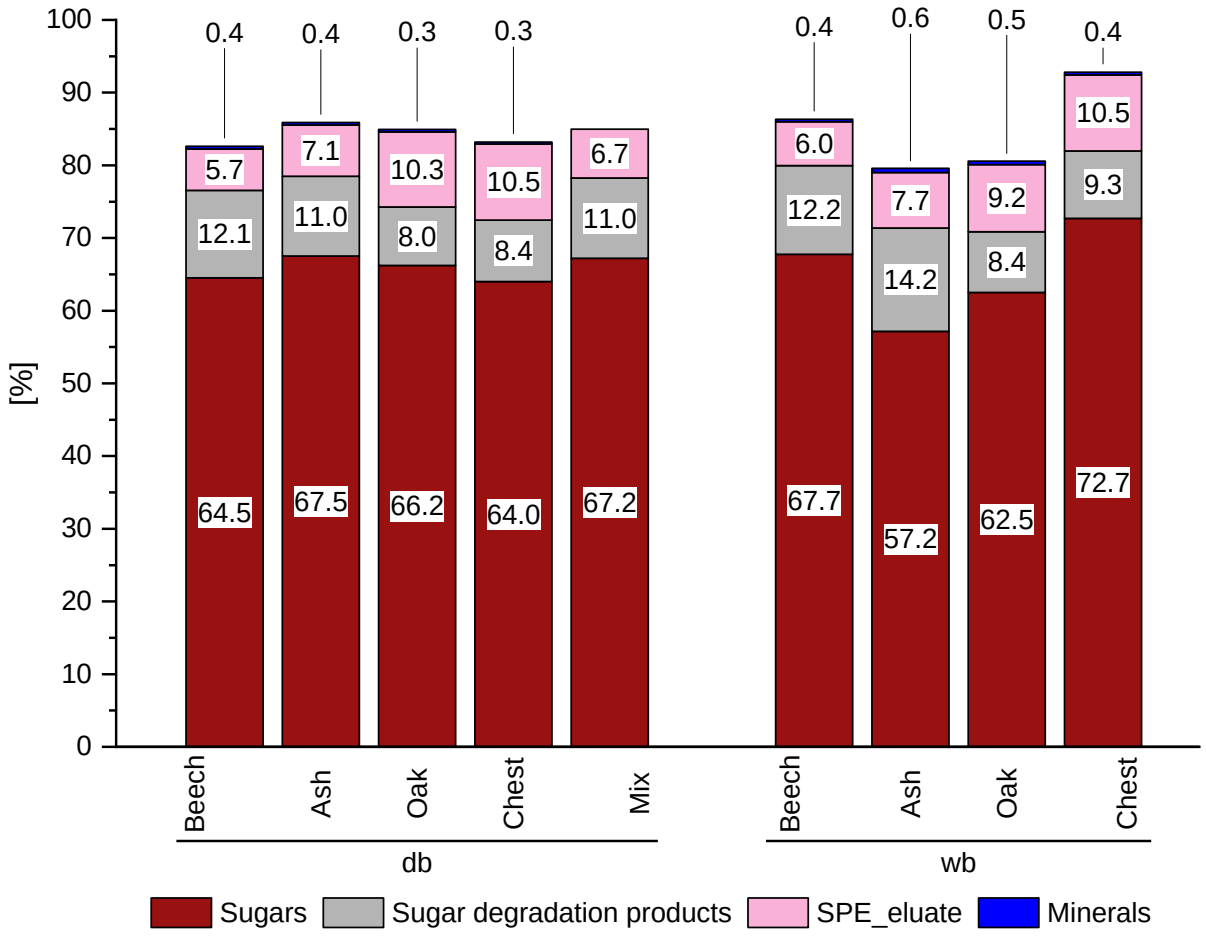


Figure 21: Mass proportions [%] of dry LPS; mineral content for the mixed sample not determined.

Part 2: Impact of factors influencing enzymatic hydrolysis and species-specific patterns

The analyses in part two mainly worked on research area 3, but were also interconnected with research area 2. The second part concentrated on research question (Rq) 3.1. This Rq focused on the evaluation of different factors influencing enzymatic hydrolysis regarding the impact severity on enzymatic hydrolysis. This comprised the evaluation of the particle size, the substrate accessibility and the S/G lignin ratio as structural aspects. Further, the influence of enzyme load, the effect of non-productive protein adsorption and the influence of inhibitory substances were evaluated, which represented interaction and mechanisms related factors. Within these analyses also the influence of bark was observed parallel, including research question 2.3. However, the analyses also touched Rq 3.2 and 3.3.

Figure 22 gives an overview on the research areas and individual Rq as well as and visualization of the analyses.

2. The second research area focused on the influence of bark on the processing of the material during pretreatment and enzymatic hydrolysis in terms of profitability and additional potentials.

2.1. How does the presence of bark influence the suitability of the tree species for the production of the main platform glucose by enzymatic hydrolysis?

2.2. How does the presence of bark influence the composition of the different process streams?

2.3. Which effect does the presence of bark have on the factors influencing enzymatic hydrolysis?

3. The third research area dealt with different factors influencing enzymatic hydrolysis and the detectable variations related to tree species-specific effects.

3.1. *How to evaluate influencing factors in terms of impact on enzymatic hydrolysis?*

3.2. *How are the tree species-specific characteristics interconnected with influencing factors on enzymatic hydrolysis?*

3.3. *Which options are available to deal with potential species-specific effects during enzymatic hydrolysis?*

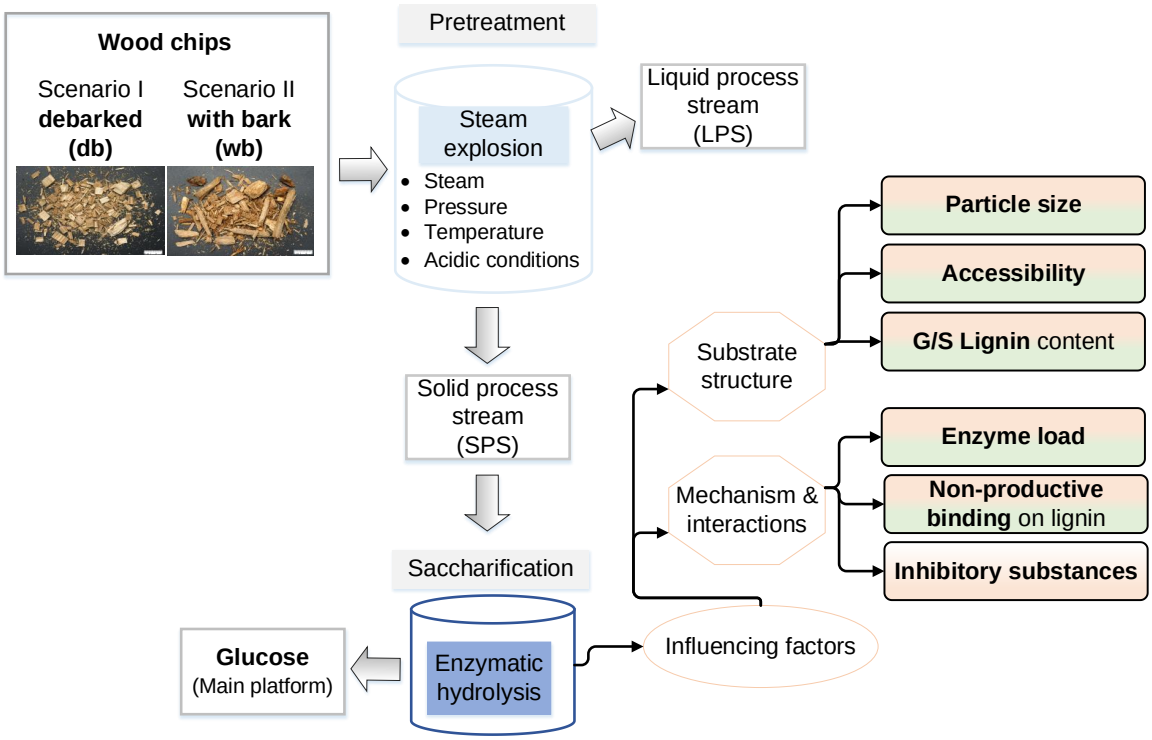


Figure 22: Overview on included analyses to work on part 2: Impact of factors influencing enzymatic hydrolysis and species-specific patterns

5.11 Particle size distribution (PSD) in the SPS

One factor, which could influence the enzymatic hydrolysis from a structural perspective, was the size of the individual particles in the SPS and the particle size distribution (PSD). The comparison of the particle size distribution allowed a **relative evaluation** between the different samples. This is based on the assumption that a decrease in particle size correlates positively with the available surface area and an increased surface area further supports the enzymatic conversion, which was also reported in literature (Shafiei et al., 2015). To circumvent effects like shrinkage the particle size was determined in wet state as described in chapter 4.2.10.

Figure 23 presents the volume weighted particle size distribution and the cumulative frequency, separately for debarked samples and samples with bark. It was observed that the **mode** ranged from 36 - 63 µm for the debarked samples and for the samples with bark from 45 - 71 µm. The mode represents the particle size with the highest volume in the sample (= peak tip). The observed small range between the mode values of the different samples led to the assumption that the process conditions were suited to reduce the particle size of all materials to a comparable level, independent to the respective tree species. However, a slight shift towards bigger mode values was observed for the samples with bark.

Interestingly, by comparing the ranges between the smallest and the biggest detected particle size the tree species showed some notable differences. For beech, oak and chestnut db the detected range was between 0.3 - 180µm, but the mode values varied with beech > oak > chestnut (db). In contrast, ash db showed a range between 0.5 - 300 µm and the mixed sample of 0.3 - 600 µm, but the respective mode values were similar to the other db samples.

The range between the smallest and the biggest particle size in combination with the form of the distribution curve described the homogeneity of the PSD, which was also represented by the **slope of the cumulative frequency**, where a high slope value indicates a better homogeneity. Following the observed values, the highest slope for the debarked samples was detected for oak with 0.92 followed by chestnut and beech as well as ash and the mixed sample, which showed the lowest slope value of 0.77. For the samples with bark the grading was chestnut > oak > beech >ash. Therefore, oak and chestnut db and wb showed the most homogenous particle size distributions (the slope was calculated between 10 and 100 µm). A table with more characteristic values to describe the PSD and the respective standard deviations was included in the appendix (Table 26, page 194).

The strongest difference between db and wb was seen for the samples of ash, as the curve of ash wb showed a second broad shoulder in the area of higher particle sizes (300 - 600 µm). A similar effect was not detected for any other species. For the same sample, the proportion of the material collected in the 2 mm sieve (see chapter 4.2.10) was notable higher than for the other samples (data not shown). From visual evaluation the residues ≥ 2 mm seemed to comprise mostly fibrous material. Therefore, the big particle proportions could be due to particles ≥ 2 mm, which could pass the sieve openings in longitudinal direction. This effect was potentially directly related to the presence of bark, as the debarked sample did not contain comparable amounts of fibrous material and did not show a second shoulder in the PSD curve. However, there was no indication that the thickness or proportion of the bark in general correlates with the size distribution as no comparable effect was detected for oak, which had a similar bark proportion than ash.

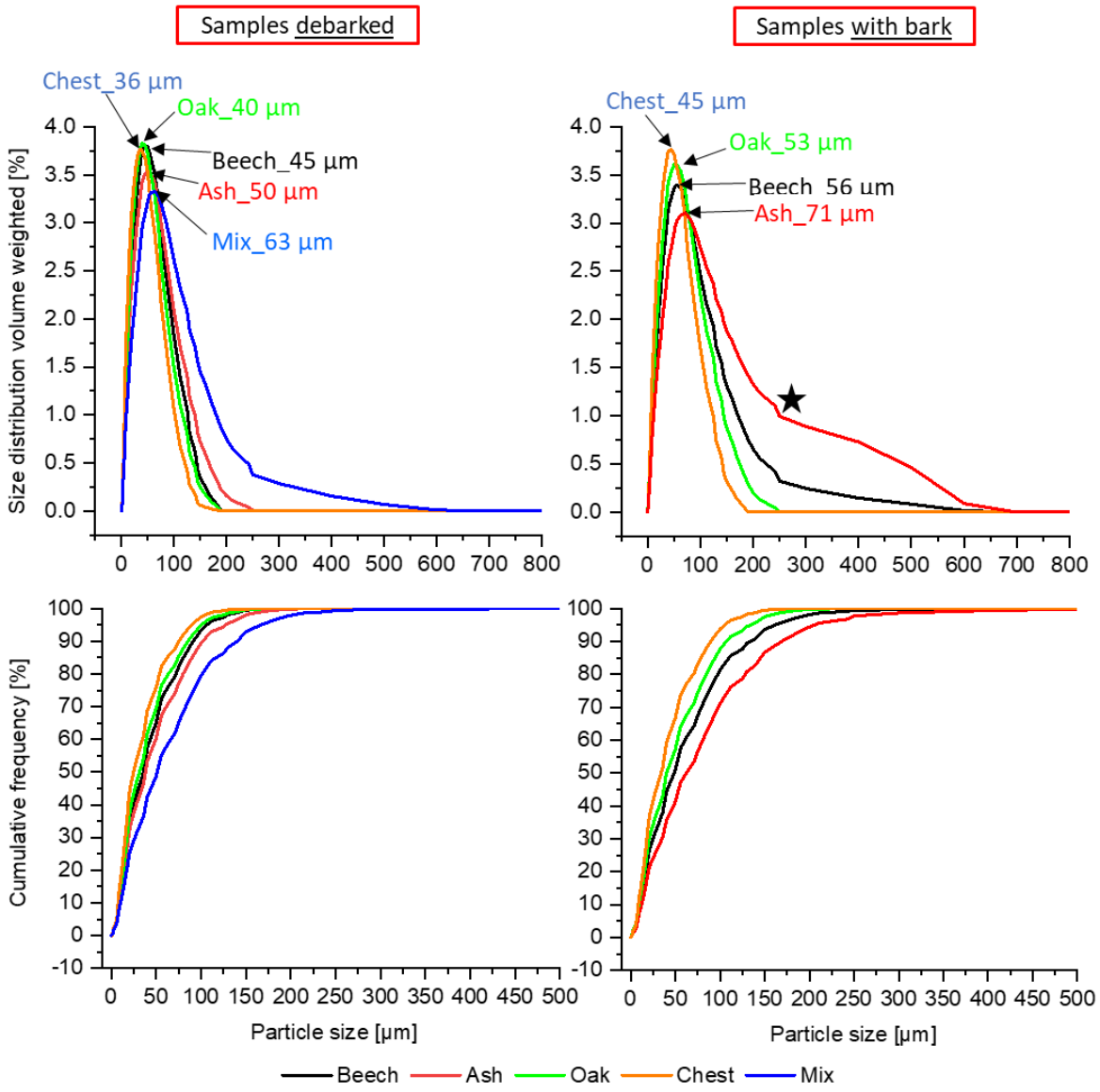


Figure 23: Volume weighted particle size distribution and cumulative frequency of debarked (left) samples and samples with bark (right); the ✶ marks the "step", which was detected in all graphs and was due to a change in the particle size scale.

The penetration of the material with water steam during the SE process was reported to be probably dependent to the conducting cell system of wood (Brownell et al., 1986; DeMartini et al., 2015). Therefore, the observed differences between the PSDs of the different samples potentially could be explained in relation to the macroscopic wood structure of each species. One theory could be that the regions with wide vessels in the early wood of ring porous species, like oak, chestnut and ash represent a "weak point" in the macroscopic wood structure. Kallavus and Gravitis (1995) also described that hardwood vessels are easily disrupted during SE and that there is a dependency towards the vessel proportion in the wood. However, the species under investigation was poplar, with a distributed pore structure. In contrast, ray cells could hold together fiber bundles, as they are less easily exploded (Kallavus & Gravitis, 1995).

In ring porous wood the early wood vessels with big volumes are located directly next to each other. In contrast, in diffuse porous woods like beech, vessels with smaller diameter are evenly spread over the cross section, embedded between other cells including fibrous cells with thick cell walls (Fengel & Wegener, 2003) and thus can probably withstand a little better the defibration forces of the explosive decompression during the SE process.

Additionally, it was reported that the secondary walls of vessels contain higher proportions of G-lignin than for the secondary walls of fibers, which contain GS-lignin. Further, it was reported that for instance parenchyma cells contain high proportions of S-lignin (Mai & Zhang, 2023). G-lignin is potentially more condensed and thus it is assumed that it is more rigid and increases the compression strength of the cell wall. This is beneficial for the vessels, which need to resist to the pressure during water transport, but at the same time a high proportion of G-lignin increases the brittleness. In contrast, a higher S-lignin proportion seems to increase the flexibility due to the lower degree of condensation (Mai & Zhang, 2023). However, differences were reported even between the vessels of ring porous and diffuse porous wood as well as between early and late wood vessels. The vessels of ring porous wood contained more GS-lignin and vessels of diffuse porous wood more G-lignin. Additionally, the early wood vessels showed thinner cell walls and contained more G-lignin than late wood vessels (Mai & Zhang, 2023).

This hypothesis of the “weak point” in combination with an probably higher G-lignin content in vessels and especially in early wood vessels (Mai & Zhang, 2023), could potentially explain the low particle size represented by the mode and the homogenous PSD of oak and chestnut. This is because, it was assumed, that the vessels have a higher compression strength, but if the forces were too high, the brittleness would cause a strong defibration of the material.

However, it has to be assumed that the year rings for oak and chestnut were smaller than for ash. This was reasoned in the correlation between the wide of the year ring and the early wood proportion in ring porous wood. With smaller year rings, the proportion of early wood and thus the proportion of vessels with big diameters is higher (Niemz et al., 2023), which would increase the fragility, following the here presented hypothesis.

This theory is supported by the fact that also the relative proportion of fiber cells certainly plays a role, as their main task is the mechanical strengthening of the wood structure. Ash for example contains ~ 62 vol% fibers, but beech just up to ~ 40 vol% (Wagenführ & Scheiber, 1974). The differences in fiber proportion and the higher S-lignin proportion in the secondary cell wall of fibers (Mai & Zhang, 2023) potentially could explain that ash db and wb showed the highest values for the mode and a broader PSD compared to beech, oak and chestnut.

Overall, these analyses showed, that the PSDs varied between the species, but small particle sizes < 300 µm took the major proportion for all samples. Additionally, there was some indication that the presence of bark increased the mode values slightly. The detected variations probably are related to the variations between the tree species regarding cell composition and distribution as well as variations in the chemical composition of the cell walls. Further, the growing conditions potentially have an influence. However, with the available data no clear evaluation of these potential effects is possible. The results of these analyses contributed to the research question 3.1 and 3.2.

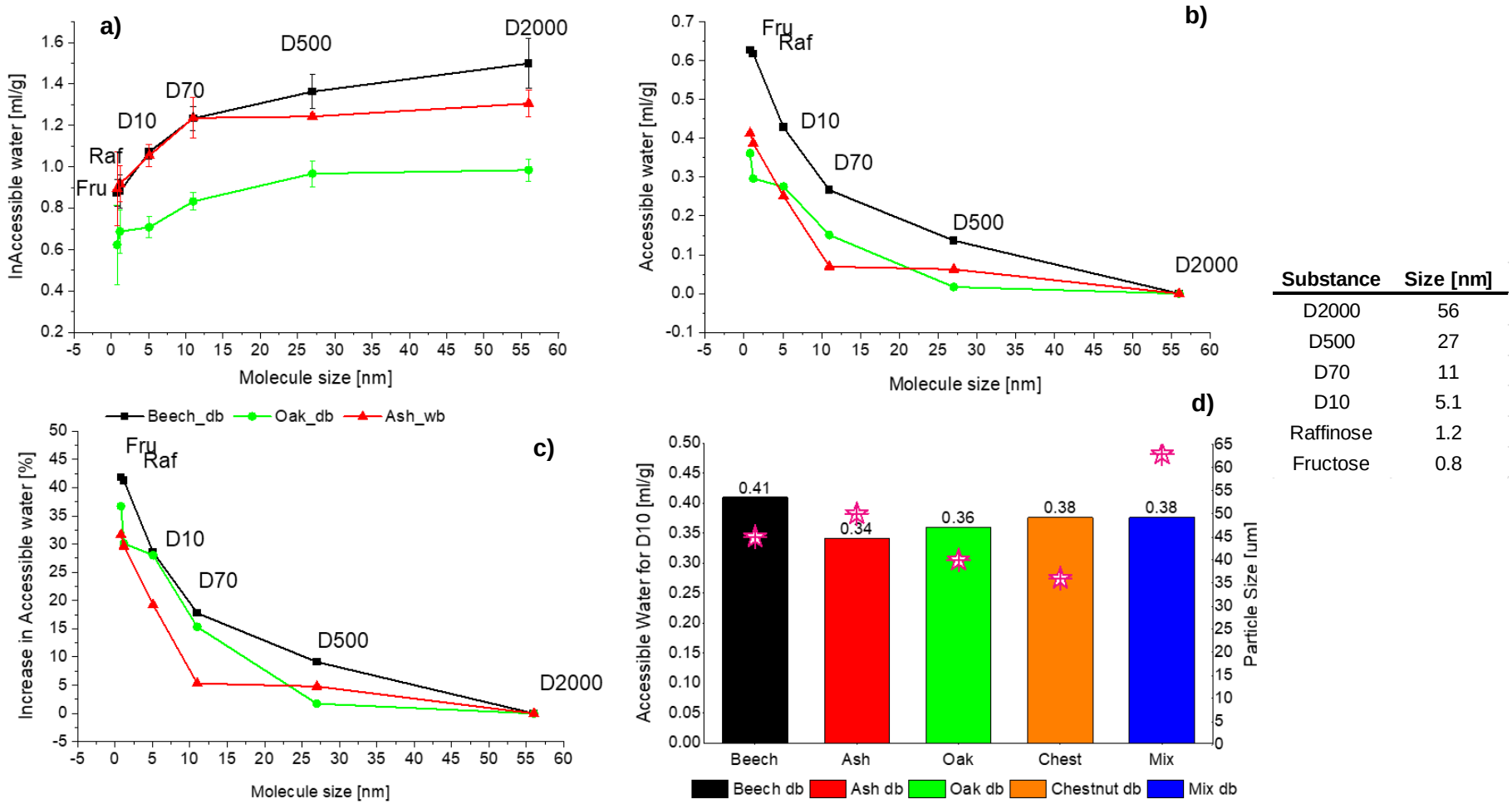
5.12 Accessibility of pores in the SPS

For the investigation of the substrate accessibility, the **solute exclusion method** was chosen (chapter 4.2.11). For this method, the wet sample is mixed with a solution containing one solute molecule of known concentration and molecular size. Through the evaluation of concentration changes it can be determined how big the accessible pore volume is (Grethlein, 1985; Wang et al., 2012). This method allowed to circumvent effects like shrinkage or hornification (Thybring & Fredriksson, 2023), which led to an underestimation of the pore volume (Beecher et al., 2009; Hill & Papadopoulos, 2001). Additionally, the solute exclusion method allowed to detect the pore volume, which is actual accessible to molecules of specific size and excludes pores, which are solely accessible by smaller molecules like water or which are closed up, with no opening to the surface (Beecher et al., 2009; Suurnäkki et al., 1997).

However, the **choice of** the respective **solute molecules** was challenging. On the one side, it had to be considered, that the molecules potentially interact with the material as described in different sources, which could influence the penetration behavior (Beecher et al., 2009; Hill & Papadopoulos, 2001). On the other side the availability of the respective solute molecules, mostly dextrans was challenging, as most manufacturers described in literature are no longer producing (see for example Grous et al. (1986)). In most cases, dextrans with similar molecular masses were available, except of the biggest dextran D2000. Grethlein et al. (1984) chose the dextran 2000 with a molecule mass ~ 2,000,000 g/mol and a size of 56 nm to define the fiber saturation point. As exactly, the same product is no longer available on the market, a substitute was chosen with comparable molecular mass, the dextran D5376 from Sigma-Aldrich with ~ 1,500,000 – 2,800,000 g/mol. For interpretation the available molecule sizes in literature were used (see chapter 4.2.11), as it was assumed that the accuracy is sufficient for the here presented approach. Nevertheless, it had to be critically considered, as the dextrans were originated from different manufacturers as the ones described in literature.

The **suitability of this method** for the respective materials was proven by different researchers (Grous et al., 1986; Ishizawa et al., 2007). For instance Grethlein et al. (1984) evaluated mixed hardwood (birch and maple) after acid hydrolysis pretreatment in a continuous plug flow reactor with the solute exclusion method applying different dextrans as well as raffinose and fructose as solute molecules. A change in the volume of inaccessible water with values of 0.51 ml/g for D2000, 0.45 ml/g for D10 before pretreatment and 0.92 ml/g and 0.41 ml/g after pretreatment were detected. The pretreatment thus led to an increase in the accessible water volume from 0.06 ml/g to 0.51 ml/g for the D10 molecule.

Regarding the **experiments performed within this thesis**, the strongest conversion discrepancy during enzymatic hydrolysis was seen between beech and oak (see chapter 5.5). Therefore, these two samples (debarked) were chosen for the analyses using the solute exclusion method. Additionally, ash wb was used, as this sample showed the most inhomogeneous particle size distribution, which potentially influence the accessibility. Figure 24 a) presents the values for the inaccessible water in relation to the molecule size of the used dextrans and sugars.



Substance	Size [nm]
D2000	56
D500	27
D70	11
D10	5.1
Raffinose	1.2
Fructose	0.8

Figure 24: a) Inaccessible amount of water for beech db oak db and ash wb; b) Calculated values for the accessible water; c) Percentage increase of accessible water volume based on the molecule size; d) Values of accessible water for the dextran D10 for all db samples (left scale), values of the mode are included and marked with pink star (right scale)

Beech db showed the highest values for the **inaccessible water** at D2000 with 1.50 ml/g, followed by ash wb with 1.31 ml/g and lastly oak db with 0.98 ml/g. This indicated that the general pore volume is higher for beech than for ash wb or oak db. With molecule size of the solutes becoming smaller, also the amount of inaccessible water is decreasing.

Interestingly for ash wb the inaccessible water is lower for D2000 and D500 as for beech db, but reaches nearly similar values for D70, D10, raffinose and fructose, which could indicate a similar pore structure in this pore size range (0.8 - 11 nm).

Further, the decrease of inaccessible water for ash wb between a pore size of 56 nm (D2000) and 11 nm (D70) showed a very low slope. Additionally, for oak db the slope between D500 and D2000 is notably lower than for beech. This probably indicated that the main pores are < 11 nm or 27 nm, respectively. The values from oak are much smaller indicating a general lower amount of pores, which would fit to the small particle sizes determined for oak.

Figure 24 b) shows the calculated amount of **accessible water** and the best accessibility was observed for beech db. The values for the accessible water for ash wb and oak were below beech db. In the study of Grethlein et al. (1984), where mixed hardwoods were analyzed, an amount of 0.51 ml/g accessible water is found for D10 after pretreatment. Compared to the here presented results, the value of beech db with 0.43 ml/g laid in the same range, but the values of oak db and ash wb clearly deviated. As discussed in the previous chapter, the species differ in their cell and pore structure, which potentially has an influence on the particle disruption during the SE process.

Figure 24 c) presents the **percentage of accessible water** based on the total amount of inaccessible water (D2000). By combining the statements of the graphs a - c) it could be seen that for beech db a maximum of 41.8 % of the total inaccessible water becomes accessible for the smallest solute the fructose (fru) with 0.8 nm. For oak db the accessible water for molecules with 0.8 nm accounted to 36.7 % and for ash wb to 31.7 %. Further, for D10 about 28.5 % of the total inaccessible water was available for beech db and interestingly also for oak db it was 28.0 %, but for ash wb it was 19.3 %. D10 has a reported size of 5.1 nm, which is in the range of the spherical size of a cellulase with 6 nm (Grethlein et al., 1984). This indicated that the theoretical proportion of pore volume, which is accessible for cellulases is the same for beech and oak db. Additionally, it was indicated that the amount of pores just accessible for smaller molecules is bigger for beech db than for oak db as the proportion of accessible water is in total higher for beech db and this sample showed the highest proportion of accessible water for fructose.

In literature a positive correlation was reported between the volume of accessible water for molecules with a size of 5.1 nm and the enzymatic conversion of pretreated poplar (dilute acid hydrolysis or steam explosion) (Grethlein & Converse, 1991). Therefore, the solute exclusion procedure was performed additionally with all debarked samples using the solutes D10 and D2000. The aim was to observe if a correlation between accessible water volume for this specific molecule size and the performance during enzymatic hydrolysis is detectable for the tree species under the present process conditions. The values were repeatedly determined for all db samples, as the method seemed to be sensitive to the mixing procedure. As artefact, the shaking device broke and had to be repaired during the experiments. Therefore, the values for D10 in Figure 24 d) are partly not identical to the first measurements. In general, it was observed that the available accessible water for molecules of an approximate size of 5.1 nm did not correlate with the different performances of the tree species during enzymatic hydrolysis. Additionally, the pink star symbols in Figure 24 d) mark the measured mode value during PSD measurements (chapter 5.11) of the samples, but no correlation between the accessibility of the D10 dextran and the most frequent particle size could be detected, either.

These analyses showed, that the method is suited for this type of material, as a decrease of inaccessible water with decreasing molecular sizes was observed, which proves consistent data (Grethlein et al., 1984). Additionally, according to the measurements the pore volume in relation to the accessible water seemed to differ between the samples. However, no obvious influence of bark or the more heterogeneous PSD for ash wb could be observed. Further, no correlation was detected between the accessibility of molecules with an assumed size of 5.1 nm and the performance during enzymatic hydrolysis. These result mainly contribute to research question 3.1 and 3.2

5.13 S- and G-lignin content of the SPS

Another structural factor potentially influencing the enzymatic hydrolysis is the lignin content in relation to the shielding of cellulose fibers. The amount of acid insoluble residues was already determined and presented in chapter 5.4, which potentially represent the lignin content as such. However, the determination of the proportions of S- and G-lignin units was assumed to reveal more information about the actual lignin structure. For the quantification of the S- and G-lignin units within the SPS a thioacidolysis in combination with GC analyses was performed.

The S-lignin units typical for hardwood differ from the G-lignin units by an additional methoxy group at the aromatic ring. Due to this additional functional group, the S-lignin is known to be less condensed than G-lignin (Mai & Zhang, 2023). It is also known that during SE pretreatment the syringyl structures are more easily cleaved and removed from the residual solid material (Li et al., 2009).

The results are summarized in Table 22. Independent to the presence of bark, db and wb samples of beech and ash, as well as the mixed sample showed a S/G ratio > 2. In contrast, for oak and chestnut a ratio < 2 was detected. These findings showed that the lignin in the SPS of oak and chestnut contained more G-lignin and beech and ash contained more S-lignin. This could be reasoned in an initially higher S - lignin content in beech and ash compared to oak and chestnut or in a stronger cleavage of the lignin linkages in oak and chestnut during the SE process. Additionally, bark had no strong influence on the ratios, which could be because the overall low bark proportions in the samples, as the wood proportion accounts to > 80%.

Table 22: Results of thioacidolysis and the present S- and G-lignin proportions.

	Proportion of degradation product [%]								
	Beech	**Ash**	**Oak**	**Chest**	**Mix**	**Beech**	**Ash**	**Oak**	**Chest**
			db				**wb**		
G-Lignin	0.93	1.00	0.86	0.85	0.92	1.03	1.15	0.91	0.76
S-Lignin	1.94	2.10	1.50	1.35	1.97	2.10	2.32	1.26	1.20
Quotient S/G	2.08	2.10	1.73	1.59	2.15	2.04	2.01	1.39	1.58
Ration S : G	**2.08 : 1**	**2.10 : 1**	**1.73 : 1**	**1.59 : 1**	**2.15 :1**	**2.04 : 1**	**2.01 : 1**	**1.39 : 1**	**1.58 : 1**
db = debarked		wb = with bark							

These results showed that the lignin structure differs between these species and that the guaiacyl derivatives take a bigger proportion in oak and chestnut. It was reported that G-lignin structures were more preferred to adsorb enzymes and that oak lignin adsorbed higher quantities of enzymes than lignin from other origins (Guo et al., 2014; Lee et al., 2022). Therefore, the increased G-lignin content in oak and chestnut, potentially could indicate a

higher enzyme adsorption capacity for these species, which probably could explain the differences detected during EH. However, this aspect as to be investigated in more detail. Different studies showed that the S/G lignin ratio was changed dependent on the chosen pretreatment and the respective intensity (Jakobsons et al., 1995; Samuel et al., 2010). Additionally, the S/G ratio can also vary within the same tree species as shown for *Populus trichocarpa*, where S/G lignin ratios between 1.3 - 4.0 were determined applying thioacidolysis (Happs et al., 2021). Therefore, the aspect of S/G lignin ratio seems to be not a completely resilient influencing factor.

These analyses showed, that the lignin structure differs between the tree species, with potential influence on the enzymatic hydrolysis. Further, it was shown that the presence of bark seemed to have no notable influence. These results comprise to research question 2.3 as well as 3.1 and 3.2.

5.14 Enzyme load and species-specific effects

Following the EH scenario 1 (see chapter 4.2.9), the SPS was converted by enzymatic hydrolysis using a defined enzyme load of 6 % based on dry material. This enzyme load was determined by pre-experiments, which tried to adapt the enzyme load to a target conversion rate of ~ 80 % for the SPS of beech db. Comparing the conversion rates of the different samples at equal enzyme/material ratio was the first approach to evaluate the suitability of the different tree species. However, it was found that this approach potentially masks some species-specific effects. The observation, that higher enzyme loads can change the interactions during hydrolysis was for instance reported by Ko et al. (2015a). It was found that at an enzyme load of 40 FPU the glucose yield increased with increasing pretreatment severity, but this effect was not observed for lower enzyme loads. Further, Sewalt et al. (1997) showed that a high enzyme load can overcome the hampering effect of lignin.

To present these observations, the EH scenario 1 was compared with the EH scenario 2, in which a defined amount of protein per gram glucan (8 mg/g) was applied. The reference glucan proportions had been determined prior to this by acidic hydrolysis (chapter 5.4). Table 23 presents the protein load for each species based on the respective scenario.

Table 23: Amount of protein within the samples following EH scenario 1 and 2; total protein content see chapter 4.2.8.

	EH scenario 1	**EH scenario 2**		**EH scenario 1**	**EH scenario 2**
Sample db	Protein per g glucan [mg/g]		**Sample wb**	Protein per g glucan [mg/g]	
Beech	15.6	8.0	Beech	15.1	8.0
Ash	15.0	8.0	Ash	17.1	8.0
Oak	16.9	8.0	Oak	20.6	8.0
Chestnut	14.6	8.0	Chestnut	17.0	8.0
Mix	15.3	8.0			
db = debarked			wb = with bark		

To enable a better comparison with other experiments, additionally the cellulase activity was determined as filter paper units (FPU). These analyses resulted in a cellulase activity of 252 FPU/ml for the enzyme blend. While this accounted for an enzyme load of 12 FPU/g glucan in EH scenario 2, the value ranged from 23 - 32 FPU/g glucan in EH scenario 1, due to the varying glucan content of the samples. However, the FPU values had to be discussed compared to values from literature. Other authors gave lower cellulase activities for the here

used cellulase blend (Cellic ®CTec2). Lee et al. (2021) reported an enzyme activity of 148 FPU/ml and Ko et al. (2015a) of 118 FPU/ml, which corresponded to a protein concentration of 190 mg/ml. Thus, a protein load of 8 mg/g glucan was equivalent to an activity of 5 FPU/ml. The differences were probably related to notable differences in the methods used for the determination of the protein content and FPU activity (compare chapter 4.2.8). Additionally, Ko et al. (2015a) used an solid load of 1 % (w/v) and in the present experiments 2 % solid load were used.

Figure 25 presents the glucan conversion after 72 h and 48 h for the EH scenario1 (similar graph for EH scenario 2 was included in chapter 5.5, page 83). Additionally Figure 26 compares the development of the glucan conversion for db and wb samples over time, applying EH scenario 1 and Figure 27 presents the respective values for EH scenario 2. The EH scenario 1 ran for 72 h, but it was found that the species-specific effects were also well describable if the run time was shortened. Therefore, following EH scenarios ran for 48 h. Nevertheless, the comparison between both scenarios revealed some effects, too, which are discussed later.

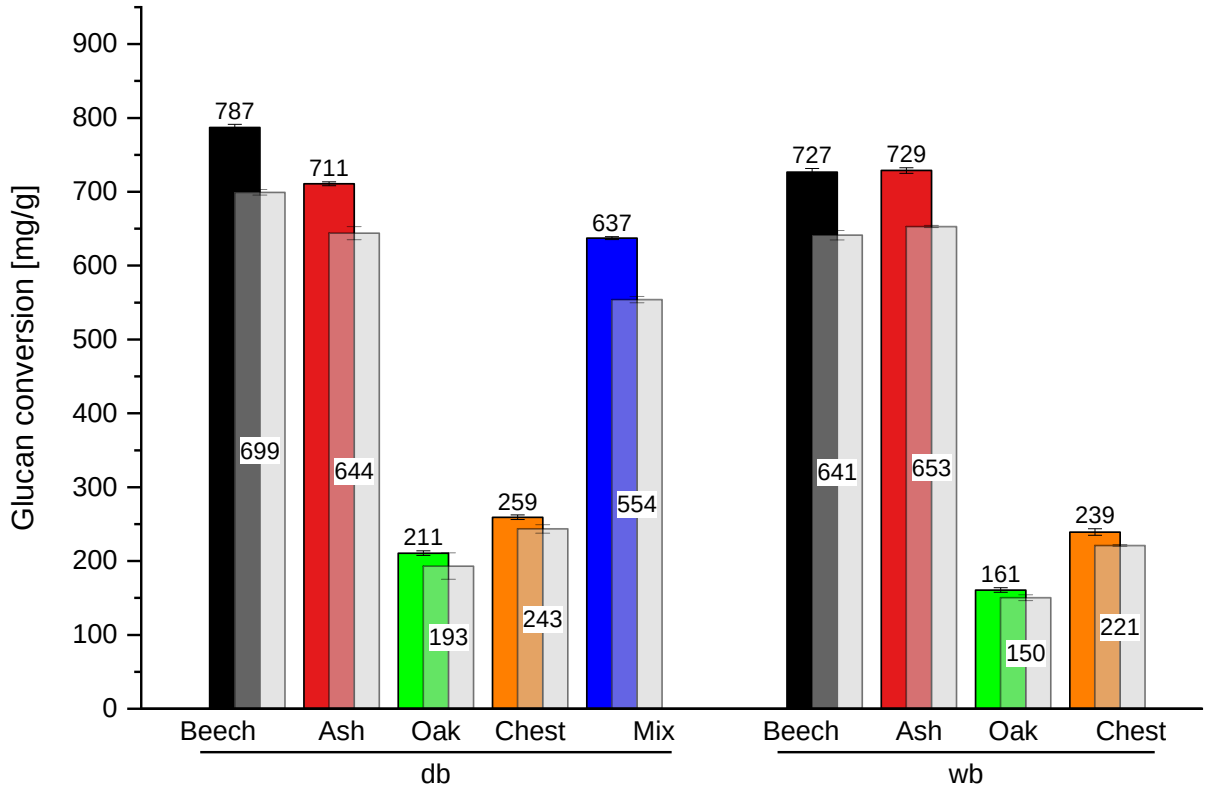


Figure 25: Glucan conversion [mg/g] during enzymatic hydrolysis following EH scenario 1; conversion after 72 h = solid bars and after 48 h = gray, transparent bars.

Regarding **EH scenario 1** with an enzyme load of 6 %, clearly beech db showed the best performance with a conversion rate of 78.7 %, followed by ash db with 71.1 % after 72 h. Interestingly, also beech wb and ash wb show a good performance with a conversion rate of 72.7 % and 72.9 %, respectively. In contrast, oak and chestnut performed worse with a conversion rate of 21.1 % and 25.9 %, respectively, for debarked samples. For samples with bark, oak showed the lowest conversion rate with just 16.1 % and chestnut showed a slightly lower conversion of 25.3 % compared to the debarked sample. The mixed sample performs in between, which strongly indicated an impact by the proportion of oak (20 %) within the mixture.

The lower conversion of oak and chestnut was constant over the whole analysis time, starting with a smaller slope for the conversion during the first 8 h compared to beech and ash (Figure 26). Additionally, the conversion seems to stagnate after 24 h for oak and chestnut. The reason for the observed decrease detected for oak between 24 h and 48 h is unclear, but is probably related with the sample taking procedure.

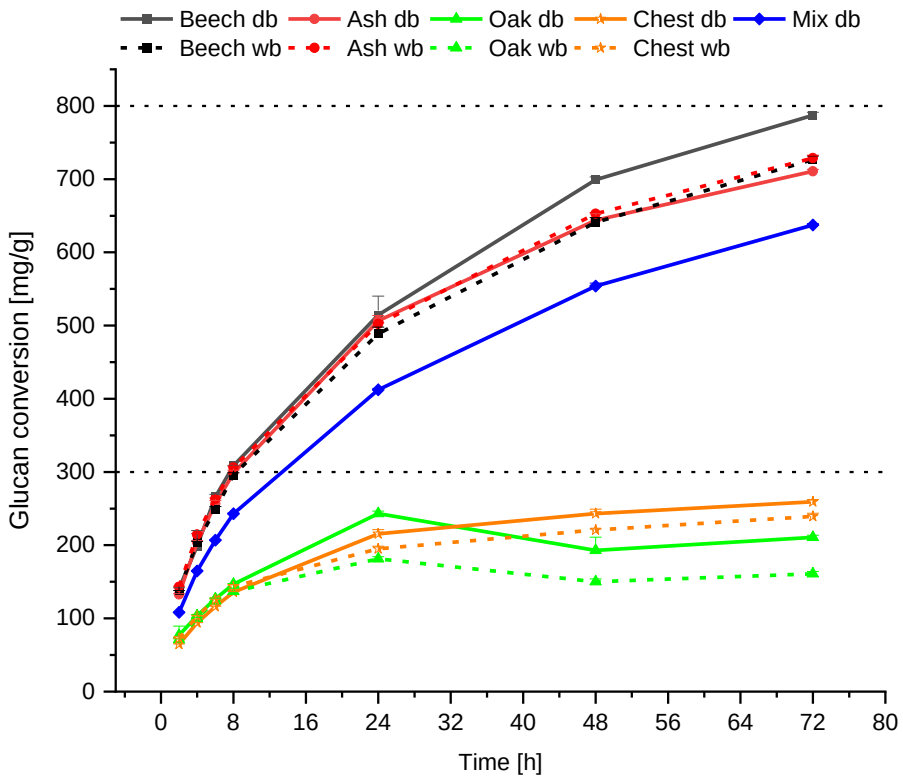


Figure 26: Glucan conversion [mg/g] in EH scenario 1 over 72 h of enzymatic hydrolysis.

Following the **EH scenario 2**, the overall picture is similar to EH scenario 1, with beech db and ash db showing the best performance with 51.1 % and 47.5 % glucan conversion after 48 h. The mixed sample performed intermediate and oak together with chestnut showed repeatedly a poor performance with conversion rates of 15.9 % and 15.2 % for the db samples and 10.6 % and 13.1 % for the wb samples after 48 h. Similar to EH scenario 1 the lower conversion for oak and chestnut could already be observed at the first sampling time point and just a very low conversion could be detected after 24 h (Figure 27).

The glucan conversion during EH scenario 1 was in all cases slightly higher after 48 h, due to a higher protein load of 14.6 - 15.0 mg/g glucan. However, considering the nearly doubled enzyme concentration in EH scenario 1 vs. EH scenario 2, the increased conversion appears low. Although it is known that there is no proportional correlation between enzyme load and conversion rate (Eckard, 2015).

Comparing both enzymatic hydrolysis approaches some **species-specific effects** could be observed. In EH scenario 2 hardly no discrepancy could be detected between beech db and wb (51.1 % db and 50.5°% wb), but within EH scenario 1 a lower conversion for beech wb was detected. In contrast, for ash db and ash wb it was the other way around. This led to the assumption, that an increased enzyme load could equalize the lower conversion of ash wb. Additionally, the results showed, that an increased enzyme load could increase the conversion of beech db, but not in the same extend for beech wb. In contrast, for oak and chestnut an

increase in enzyme load led to a slightly increased conversion, but similar for both species and not dependent on the presence of bark.

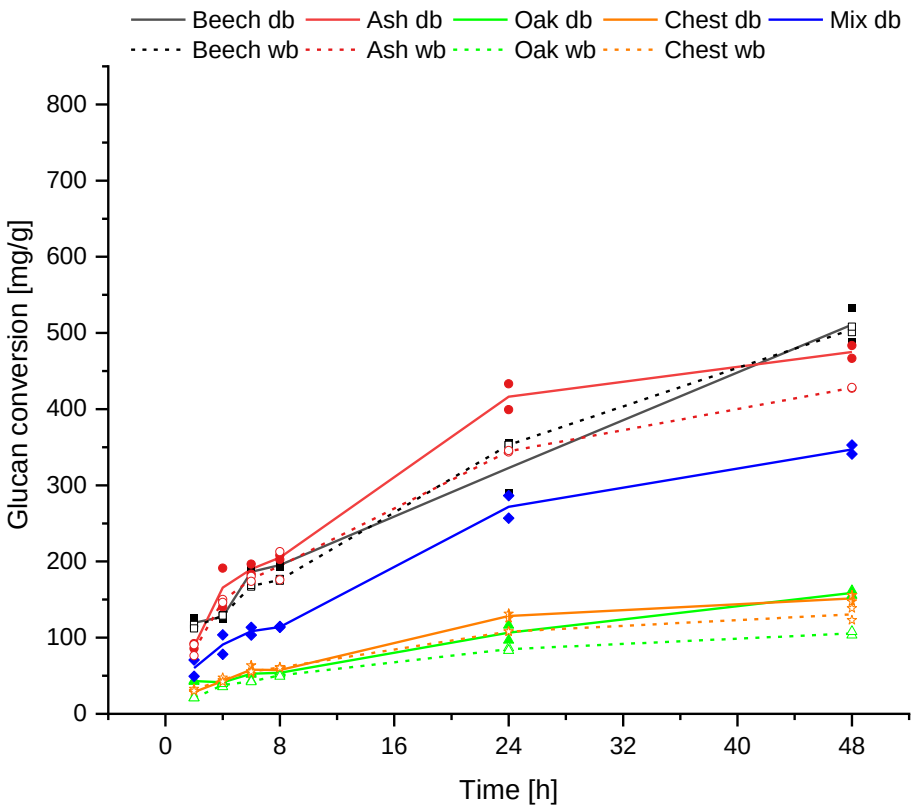


Figure 27: Glucan conversion [mg/g] in EH scenario 2 over 48 h of enzymatic hydrolysis; lines represent mean values and symbols the respective individual values.

Seidel (2019) determined an enzymatic cellulose digestibility of 6.9 - 99.1 % during the experiments with pretreatment conditions of 7 - 27 barg steam pressure and time variations between 5 and 15 min. Enzymatic hydrolysis was performed with 1 % w/w cellulose and 60 FPU/g cellulose at 50 °C for 120 h. Cotana et al. (2015) found an enzymatic hydrolysis yield of 40.5 % - 97.82 % for *Q. ilex*, 48.4 - 94.8 % for *Q. cerris* and 42.03 - 96.3 % for *Q: pubescens*, dependent on the respective conditions during SE pretreatment and enzymatic hydrolysis. The enzymatic hydrolysis was performed for 48 h with enzyme loadings from 7 - 13 % based on dry matter, underlining the strong dependence of the yield on the applied process conditions.

These examples from literature show, that both approaches to set the enzyme load were used. Additionally, it verifies that there are several other factors influencing the enzymatic hydrolysis, which potentially level effects of varying enzyme concentrations. However, the orientation of the enzyme concentration based on the glucan content seemed to be beneficial for the investigation of species-specific effects. These analyses thus contribute on the one side to research question 3.1 and 3.2 and on the other side the results were used as reference values for comparison during following experiments.

5.15 The non – productive adsorption of enzymes

In literature, various factors influencing enzymatic hydrolysis were investigated, like the accessibility of the substrate, the cellulose crystallinity or inhibitory effects of degradation products (Grethlein, 1985; Kim et al., 2011; Puri, 1984). One influencing factor, which was considered to be important was the non-productive binding effect. This effect describes the long-term adsorption of enzymes to the substrate, which are thus no longer available for reactions. In most studies this adsorption effect was attributed to lignin, but was observed for pure cellulose, too (Eckard, 2015; Yuan et al., 2021). It was found that the non-productive adsorption varies and was dependent to the substrate and pretreatment type, which both have an influence on the lignin structure (Ko et al., 2015a; Lee et al., 2022).

Different surfactants and the cattle protein Bovine serum albumin (BSA) were used as additives during enzymatic hydrolysis to reduce the necessary enzyme load and increase the conversion as well as to investigate the lignin-enzyme interaction mechanisms. The detected positive effects were mostly attributed to a reduced non-productive binding of enzymes to the lignin (Eriksson et al., 2002; Yang & Wyman, 2006).

The concept of surfactants improving enzymatic hydrolysis was suggested by several studies (Eriksson et al., 2002; Wang et al., 2020; Zhang et al., 2018). However, the application was rarely used to investigate species-specific effects, especially for hardwoods and the species beech, ash, oak and chestnut, which were examined here. A further new aspect was the investigation of the effect of bark within this context.

Based on literature, non-ionic surfactants worked best for pretreated lignocellulosic material, which is similar to the herein used SPS after steam explosion. Under consideration of environmental aspects, Tween20 was chosen over other non-ionic surfactants such as Triton. This was because Tween20 does not contain an aromatic ring, but is a polyoxyethylene sorbitol ester. Additionally, BSA was applied as an alternative additive, which is chemically different to surfactants, but was observed to lead to the same effects (Eriksson et al., 2002; Ooshima et al., 1986; Sivars & Tjerneld, 2000; Yang & Wyman, 2006).

Figure 28 presents the glucan conversion of beech db over a time period of 48 h represented by different sampling times and compares the control scenario (EH scenario 2) with the experiments including the additives BSA (EH scenario 3) or Tween20 (EH scenario4) (chapter 4.2.9). Both additives showed a positive effect and led to an increased glucan conversion. This enhancement was detectable directly from the beginning of the reaction (first sampling after 2 h). The strongest increase was observed for Tween20. For beech db the conversion was enhanced by about +14.9 % for BSA and about +35.0 % if Tween20 was used as additive.

Figure 29 presents the respective results for ash db, which showed a positive effect for both additives, similar to beech db. However, the effect seemed not to start until 4 – 6 h of the reaction and the discrepancy between BSA and Tween20 became slightly stronger after 24 h.

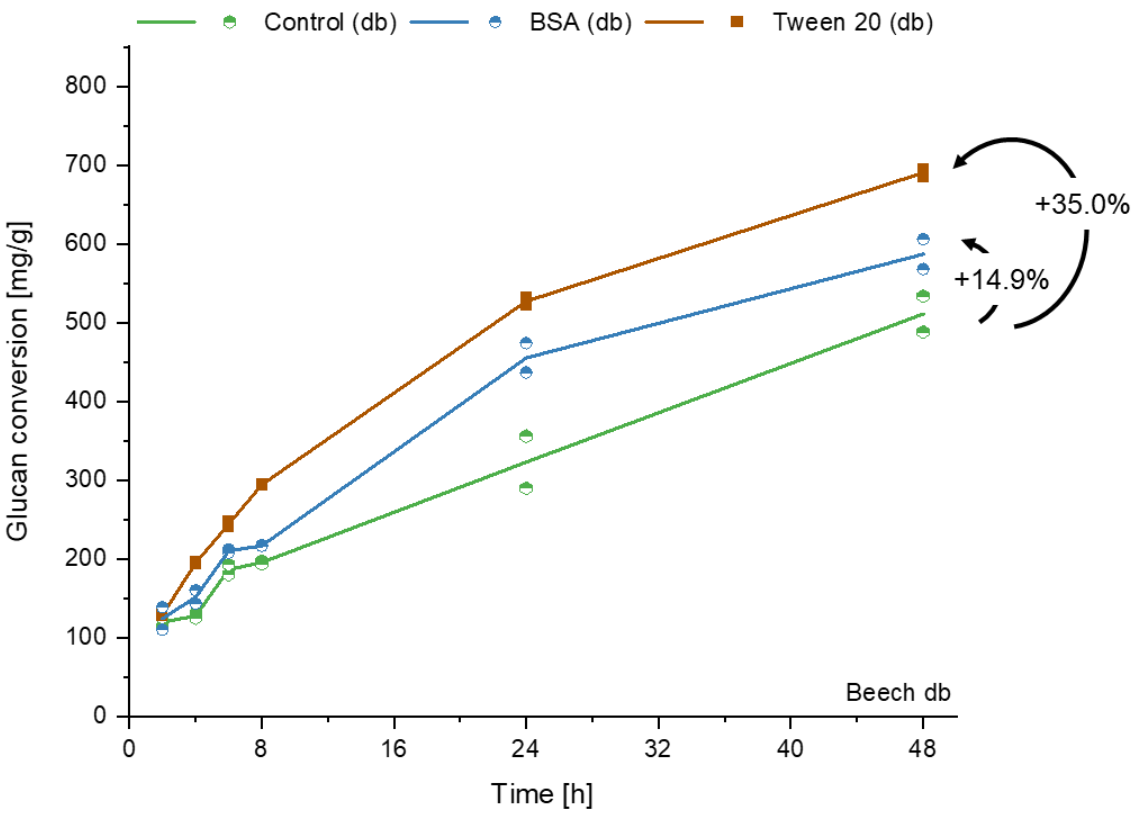


Figure 28: Glucan conversion [mg/g] over time with beech db comparing three different EH scenarios (2, 3 and 4); mean values are presented as solid lines and individual measurements as symbols.

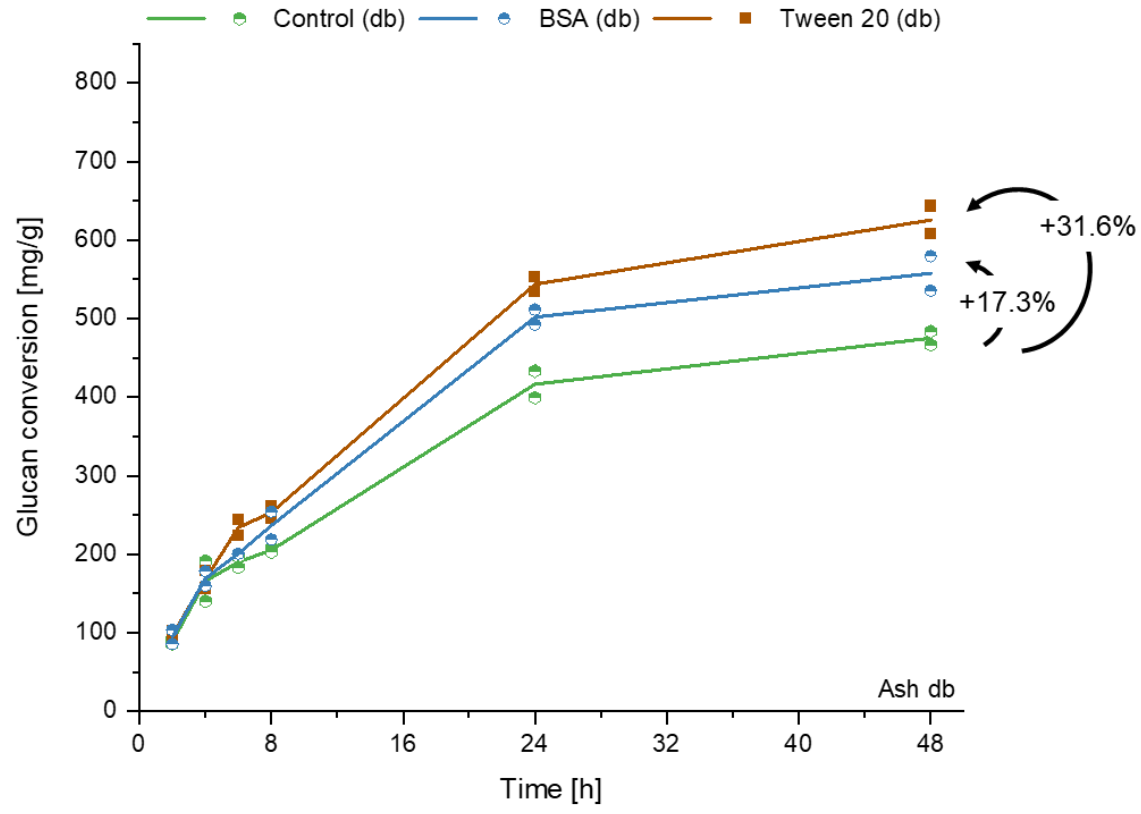


Figure 29: Glucan conversion [mg/g] over time with ash db comparing three different EH scenarios (2, 3 and 4); mean values are presented as solid lines and individual measurements as symbols.

During the enzymatic hydrolysis of beech db following EH scenario 1, a conversion rate of 78.7 % was detected after 72 h. In the here presented experiment using Tween20, 69.0 % of glucan was converted already after 48 h with approximately half of the protein load (EH scenario 4). The same effect can also be seen for ash db. This indicated that Tween20 could be used to enhance the conversion rate or to reduce the reaction time. These results were in line with findings from literature, in which the use of surfactants led to an increased conversion, or the option to reduce the applied enzyme load by ~ 50 % (Eriksson et al., 2002).

Figure 30 presents the results for the mixed sample db containing 60 % beech and 20 % ash and oak, respectively. For this sample, the effect of BSA and Tween20 was detectable from the beginning of the reaction. The discrepancy between the additives seemed to stay constant over the whole reaction time. However, the observed increase of the conversion rate was notably higher for the mixed sample with +33.3 % (BSA) and +60.2 % (Tween20) compared to beech and ash db. This was unexpected as the content of beech and ash accounts for 80 % of the mixed sample composition and indicated that by far the strongest effect was derived from a released inhibition of the (minor) oak substrate.

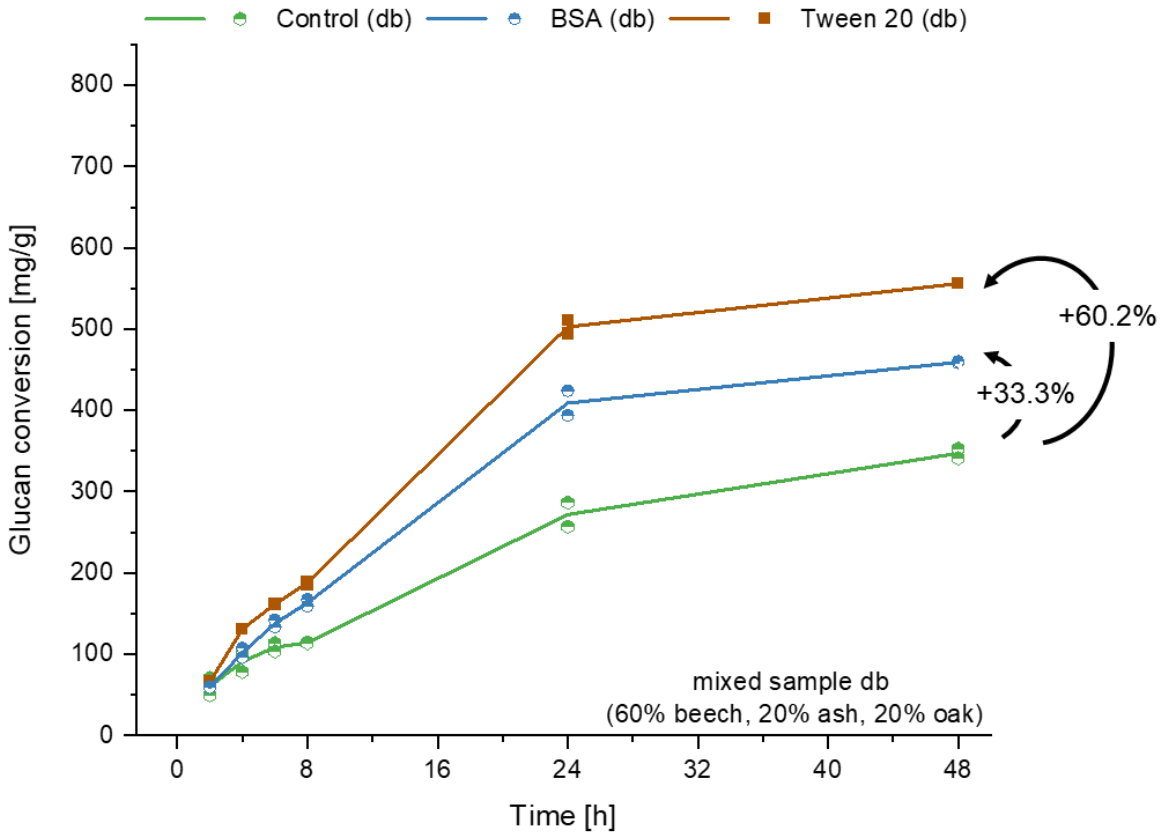


Figure 30 Glucan conversion [mg/g] over time with the mixed sample db comparing three different EH scenarios (2, 3 and 4); mean values are presented as solid lines and individual measurements as symbols.

The evaluation of the experiments for oak and chestnut (Figure 31 and Figure 32) confirmed this assumption, since the detected increase was in a much larger scale compared to the earlier results with ash and beech. With BSA, the conversion was improved by +106.3 % for oak and +120.4 % for chestnut, which was surpassed by Tween20 with increases of +244.7 % and +267.8 %, respectively.

The enhanced conversion was detected directly from the beginning of the reaction, but the discrepancies between BSA and Tween20 increased after 8 h. After this sampling time the BSA conversion curve shows a much lower slope than the Tween20 curve, similar to the control, but on a higher conversion level. This indicated that the positive effect of BSA was more pronounced in the beginning of the reaction, but the effect of Tween20 lasted longer.

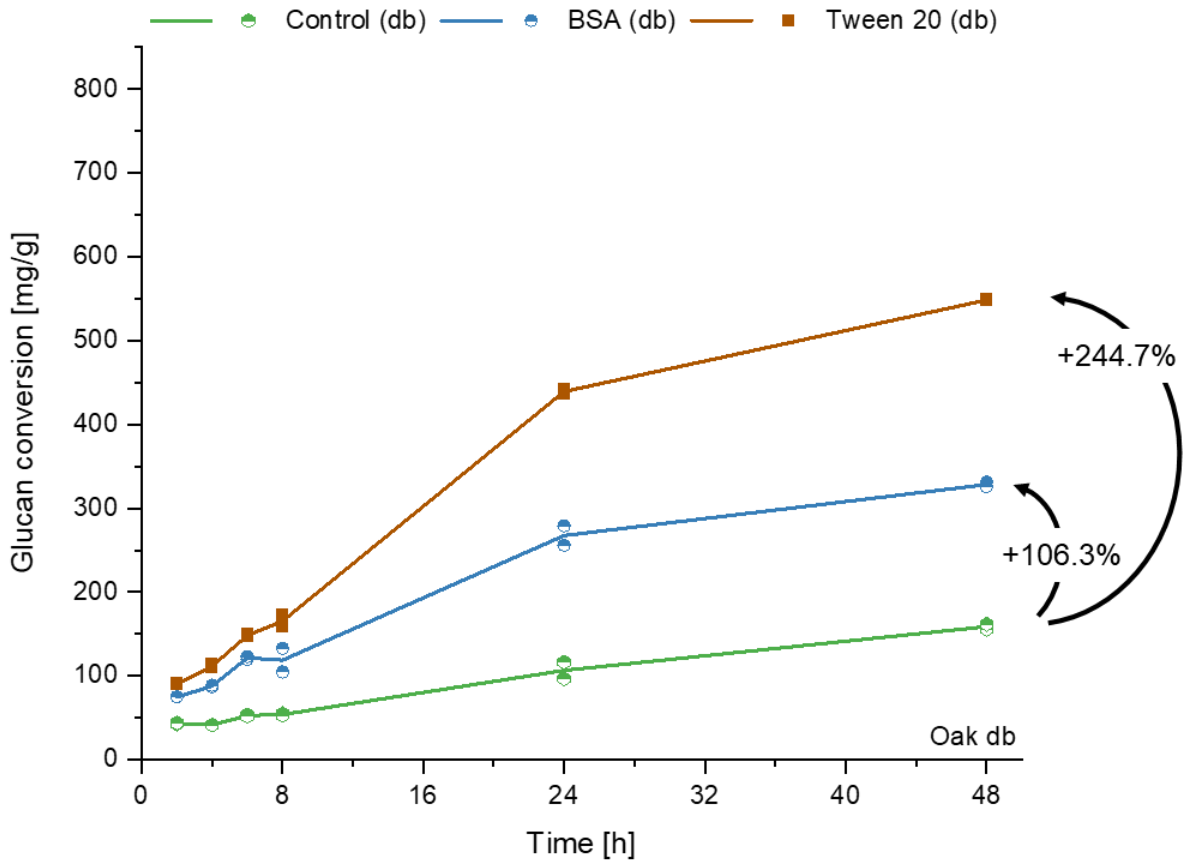


Figure 31: Glucan conversion [mg/g] over time with oak db comparing three different EH scenarios (2, 3 and 4); mean values are presented as solid lines and individual measurements as symbols

Yang and Wyman (2006) could improve the enzymatic conversion (at 20 FPU/g) of steam exploded Douglas fir from 54.2 % to 73.5 % (~ +36 %) after adding 1 % of BSA to the reaction. These results are higher than the here detected values of +16 % or +17 % for beech and ash, but are in the same range as for the mixed sample. For steam pretreated spruce, a conversion of 33 % was reported without an additive. This value could be increased by the addition of non-ionic surfactants to conversion rates of 43 - 48 % (Eriksson et al., 2002). This range of improvement due to non-ionic surfactants was in line with the improvements detected in this thesis for beech, ash and the mixed sample. In contrast, the results for oak and chestnut surpassed the reported results from literature.

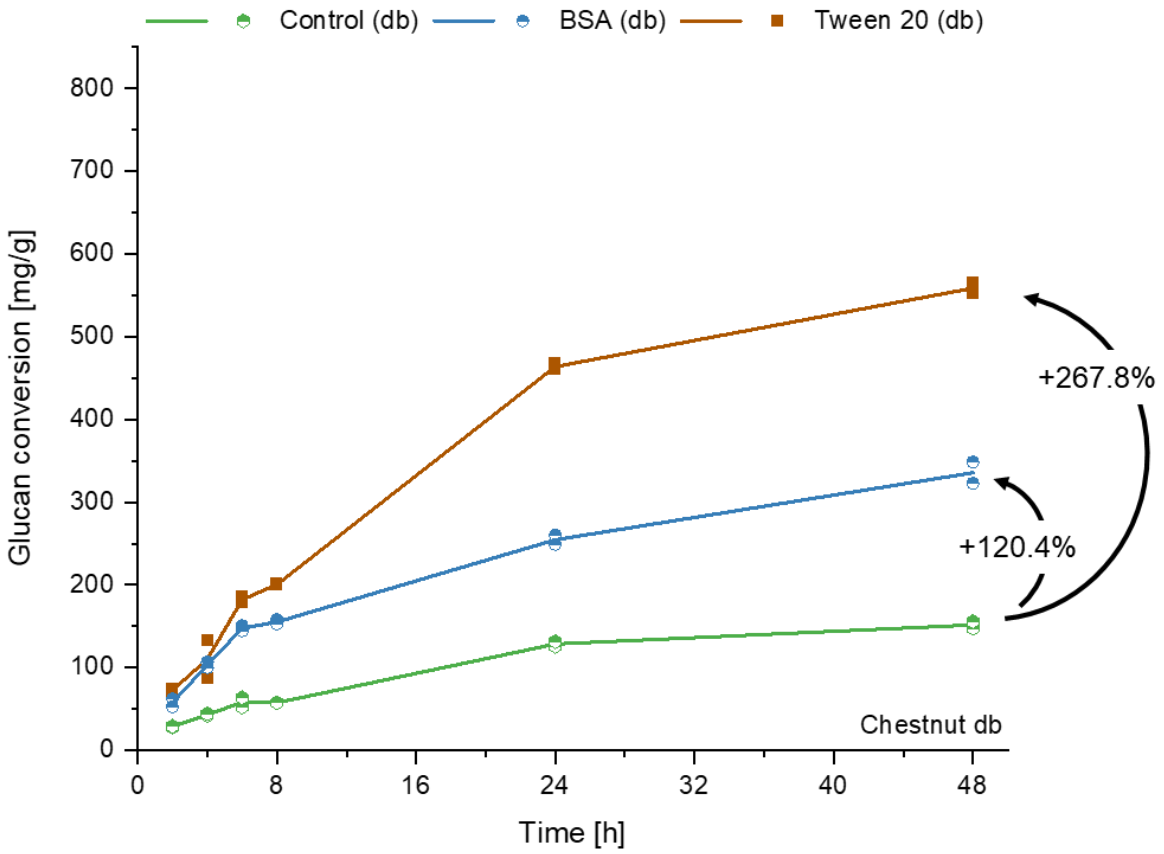


Figure 32: Glucan conversion [mg/g] over time with chestnut db comparing three different EH scenarios (2,3 and 4); mean values are presented as solid lines and individual measurements as symbols.

Figure 33 presents the results for the same experimental setup, but comparing the samples with bark with the debarked samples to discuss the influence of bark. In all cases, a strong to slightly negative effect was observed if bark was present in the sample material. Interestingly, this seemed to correlate with the bark proportions of the tree species.

The presence of bark reduced the conversion rate just slightly for beech and chestnut. For beech, the samples with bark showed very similar conversion rates in the control and the BSA scenario constantly over the whole reaction time. Solely for the Tween20 scenario, a slightly lower conversion was detected during the first 24 h, but reached nearly the same final conversion rate after 48 h with 69.0 % (db) and 67.5 % (wb). For chestnut the presence of bark reduced the conversion rate slightly more than in the case of beech at least for the control and the BSA scenario, but in the Tween20 scenario, the sample with bark reached the same conversion rates after 48 h with 55.6 % (db) and 56.5 % (wb). Regarding the bark proportion, beech and chestnut were the species with the lowest bark proportions of 6.7 % and 8.1 % (see chapter 5.1)

For ash, the discrepancy between db and wb was strongest for the BSA scenario, where the conversion was detected to stagnate completely after 24 h. For oak the samples with bark had a lower conversion in all scenarios and the negative discrepancy between db and wb was detected to start after 8 h of enzymatic hydrolysis, like it was the case for the other species, too. The negative effect of bark for ash and oak samples was found to be stronger compared to beech and chestnut and was most enhanced in the BSA scenario. The bark proportions for oak and ash were estimated with ≥ 15 %. The conversion rate for oak db applying Tween20 is 67.1 % higher than the BSA - related value, but for the sample with bark the conversion rate of the Tween20 reaction was 117.7 % higher than for the BSA scenario.

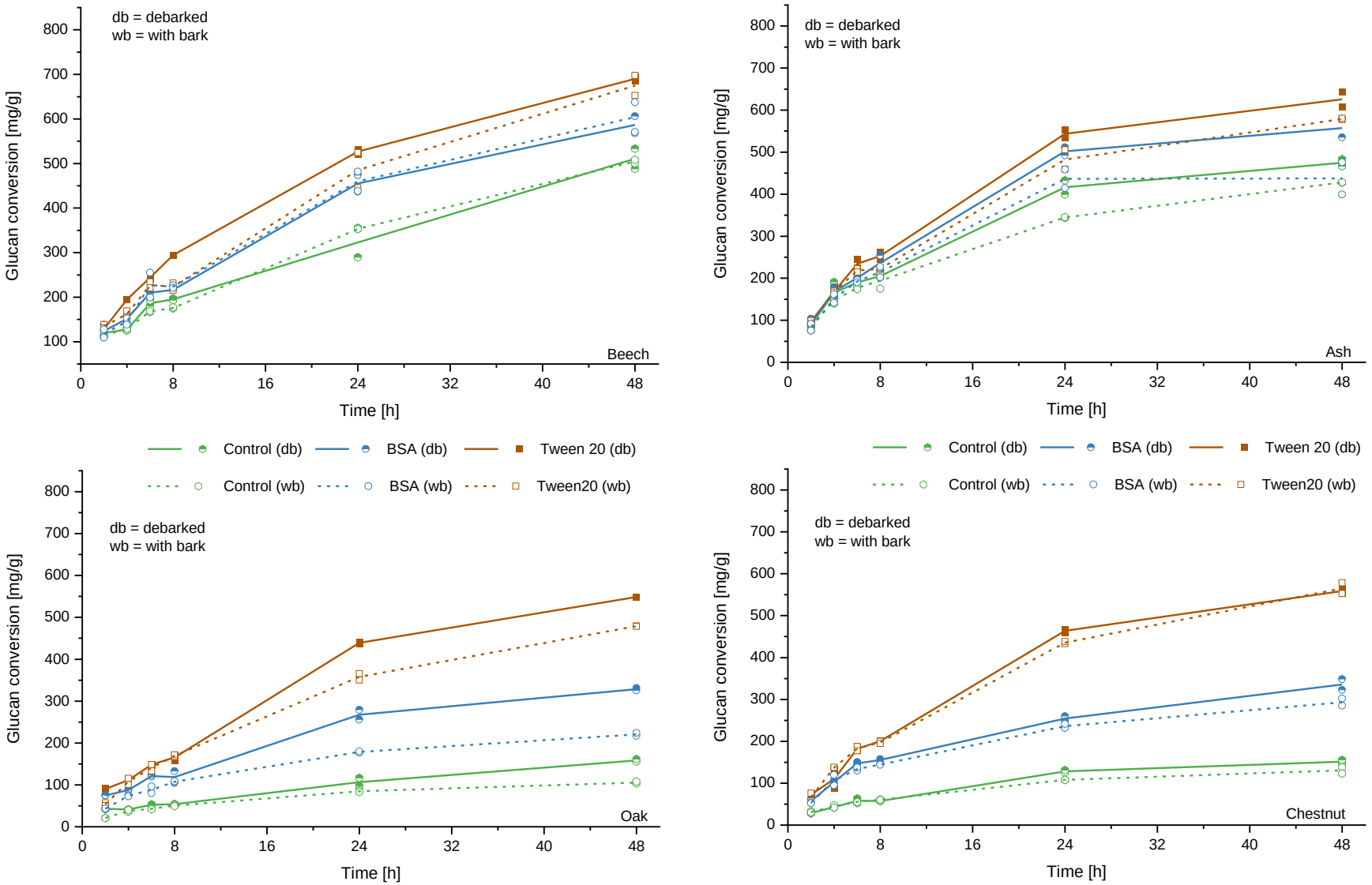


Figure 33: Glucan conversion [mg/g] over time with beech, ash, oak and chestnut wb and db comparing three different EH scenarios (2,3 and 4); mean values are presented as solid lines and individual measurements as symbol.

Figure 34 presents the total glucan conversion after 48 h of enzymatic reaction for the debarked samples and samples with bark for the different scenarios with BSA and Tween20 as well as the reference.

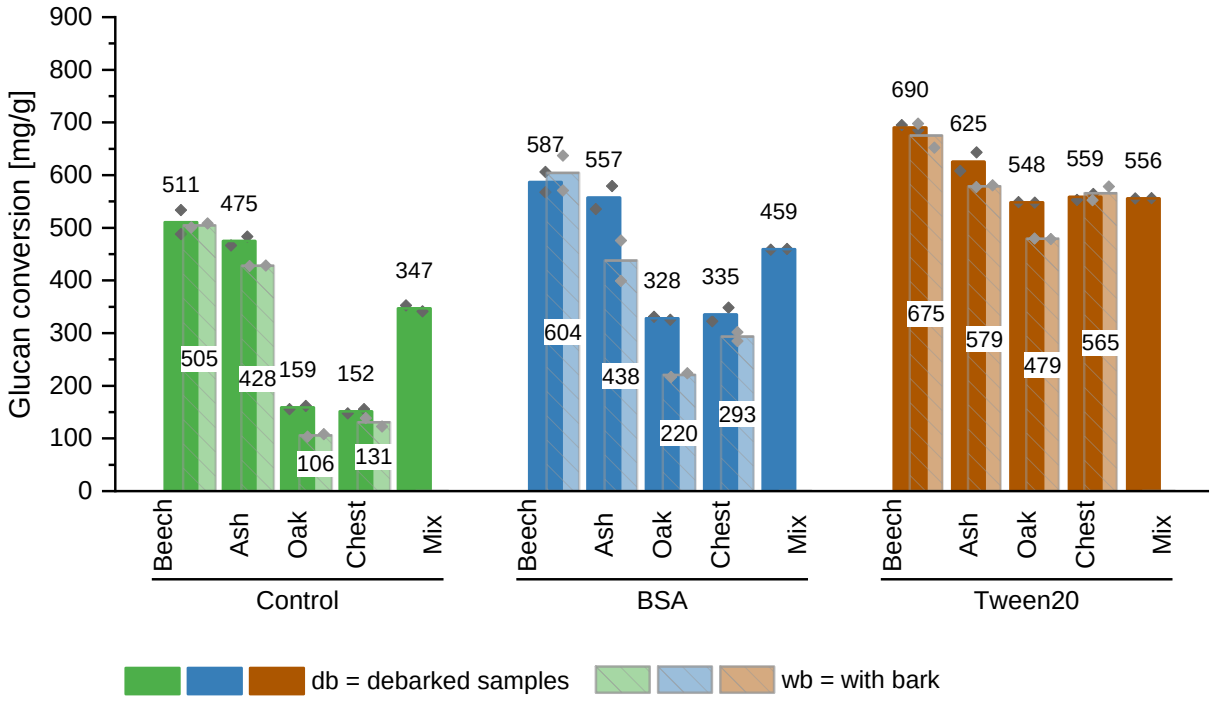


Figure 34: Glucan conversion after 48 h of enzymatic hydrolysis for beech, ash, oak and chestnut debarked and with bark; bars represent the average values and the symbols represent the individual measurements.

The additive BSA showed a smaller positive effect than Tween20 and the presence of bark influenced the performance of the additives differently. This indicated that Tween20 and BSA had different mode of actions, which is reasoned in the molecular structure. Tween20 is a non-ionic surfactant, which has a polar and unpolar molecule part. The effect of Tween20 was described to work at the solid-liquid interface, where the surfactant shows a higher affinity towards lignin than polysaccharides. The interaction of the ethylene oxide chains with the lignin surface is assumed to happen by hydrogen bonds. The interaction with lignin is preferred due to stronger hydrogen bonds with phenolic hydroxyl groups than with hydroxyl groups of polysaccharides (Kaiser, 2018; Seo et al., 2011). It was also reported that Tween20 performed better for intense pretreated materials or for acid pretreated materials then for material, which was pretreated by an oxidative sodium chlorite pretreatment (Chen et al., 2018) Transferred to the observed effects, the described interactions with lignin would also explain the constant positive effect of Tween20 over the whole reaction time.

The mechanism behind the interaction of BSA with lignin can probably be described as a “dummy-effect”. Therefore, the BSA basically competes with the cellulase enzymes for the potential adsorption areas at the lignin (Yang & Wyman, 2006). In contrast, Tween20 also occupied these adsorptive areas, but is not exchangeable by the enzymes. As it had to be assumed that BSA was solely adsorbed to the lignin surface, the reduced positive effect of BSA over time, could be explained by a higher affinity of the lignin towards the actual enzymes. The BSA and the Tween20 were added prior to the enzyme solution, thus BSA potentially occupied the respective sites at the lignin surface. However, after addition of the enzyme solution it was slowly exchanged or an adsorption equilibrium between BSA and the enzymes established. Additionally, it could be possible, that the BSA did not occupy all adsorbing areas

on the lignin and with increasing reaction time, the possibility increased that the enzymes in solution found an adsorbing area, which was still free. Regarding the affinity of lignin to adsorb enzymes, it was reported that the lignin structure, as well as the enzyme producing organism can influence the affinity (Chen et al., 2018; Ko et al., 2015b; Rahikainen et al., 2013b).

To investigate the aspect of **protein adsorption** further, the final protein concentration in the supernatant after 48 h of enzymatic hydrolysis was measured by total nitrogen analysis (compare chapter 4.2.8). For the control and Tween20 scenarios the initial protein concentration with 8 mg protein per gram glucan was 0.1 mg/ml (work volume was 200 ml). For the BSA scenario, the added BSA concentration had to be considered as well and thus the initial protein concentration was 1.1 mg/ml. Considering, the initial protein amount, the reduction of free protein within the supernatant was calculated and the values are presented in Table 24.

Table 24: Decrease in free protein in the supernatant after 48 h of enzymatic hydrolysis.

Sample	Decrease of free protein [%]			Sample	Decrease of free protein [%]		
db	Control	BSA	Tw20	wb	Control	BSA	Tw20
Beech	-63	-65	-29	Beech	-67	-66	-27
Ash	-65	-55	-34	Ash	-58	-68	-29
Oak	-80	-95	-44	Oak	-70	-96	-27
Chestnut	-82	-95	-50	Chestnut	-78	-96	-42
Mix	-71	-73	-40				
db = debarked		wb = with bark					

First focusing on the **control** with no additive, it was observed that less than 40 % of the protein remained for beech, ash and the mixed sample, but for oak and chestnut solely less than 20 % of the original concentration was detected.

Interestingly, the samples of oak and chestnut with bark showed slightly less enzyme adsorption than the debarked samples with 30 % and 22 % of remaining free protein in the supernatant. The results of the control scenario indicated, that the adsorption capacity of oak and chestnut was substantially higher than for beech and ash, partly explaining the lower conversion rates. A dependency of the adsorption affinity towards the raw material was also reported by different researchers (Lee et al., 2022; Rahikainen et al., 2013b).

An adsorption affinity towards specific enzymes like ß-glucosidases could not be assumed at this point. For instance, raised amounts of cellobiose could be a hint to an increased adsorption of ß-glucosidases, which could lead to product inhibition as well. (Ko et al., 2015b), but no increased cellobiose contents were observed.

For the **BSA scenario**, with a protein concentration of 1.1 mg/ml, the decrease of free protein stayed in the same range for beech and ash compared to the control. However, since ~90 % of the total protein in these samples is represented by BSA, this indicates that the increased glucan conversion was based on a successful competition by the BSA proteins, resulting in a higher proportion of active enzymes in solution. The lower conversion rates compared to Tween20 were probably due to the exchange of adsorbed BSA with enzymes over time.

However, for oak and chestnut, the decrease of free protein in the supernatant was observed to be stronger than in the control scenario, with less than 10 % of the proteins remaining in solution. This strongly supports the assumption, that the protein adsorption capacity of oak and chestnut substrate was notably higher than for beech and ash. Interestingly, within the BSA

scenario no difference in enzyme adsorption could be detected between oak and chestnut db and wb.

The **Tween20 scenario** increased the amount of free enzymes in the supernatant notably. For all debarked samples the enzyme adsorption was reduced to about half compared with the control. For the samples with bark, the enzyme adsorption was half compared to the control, too. One exception was oak wb, where the adsorption decreased stronger with 73 % of the proteins remaining in the Tween20 scenario compared to 30 % in the control. Eriksson et al. (2002) also detected a doubled content in free enzymes after the addition of Tween20. This is also the case for oak and chestnut and explained the strong positive effect of Tween20 for these tree species.

The measurement solely included the free protein within the supernatant. Therefore, for the interpretation, it had to be considered that the detected reduction in free protein could include different mechanisms. First, the productive binding of enzymes for the active conversion of cellulose, as after 48 h the available cellulose was not converted to glucose completely (Sun & Cheng, 2002). Second, the already discussed non-productive binding to the substrate surface (Eriksson et al., 2002). Third, the enzymes probably were inactivated and precipitated or absorbed by the substrate (Ximenes et al., 2011) and thus probably irreversibly inactivated.

In the case of oak and chestnut, it is known that these tree species contain high amounts of **hydrolysable tannins**. This agreed with the detected proportions of gallic and ellagic acid during the previously presented measurements (see chapter 5.7 and 5.8). Therefore, it was assumed that the high protein adsorption capacity of oak and chestnut was just partly reasoned in the interaction of enzymes with lignin, but was also due to protein-tannin interactions.

Hydrolysable tannin can form tannin-protein complexes by hydrophobic interactions as well as hydrogen bonds. These complexes bind the enzymes and the respective complexes can stay dissolved or precipitate dependent to the solubility of the complex (He et al., 2006; McManus et al., 1985). This binding by tannins could be non-productive and contribute to the here detected reduction in free protein if the protein-tannin complexes were precipitated. If the tannin-protein complexes were still soluble, they should have been detected in the total nitrogen measurements. The non-productive binding effect of tannins was found to be reversible for instance by adding a surfactant (Goldstein & Swain, 1965; Gustavson, 1954; Oh et al., 1980). These mechanisms could explain the additional protein adsorption capacity of oak and chestnut, as well as the strong positive effect of Tween20. Further, an enhanced adsorption of BSA protein by hydrolyzed tannins was reported by He et al. (2006), which could also explain the high reduction of free protein for oak and chestnut in BSA scenario.

Overall, these experiments showed that both additives could increase the conversion, with a stronger and more continuous effect for Tween20. An experimental setup applying both additives would be one approach to clarify the mechanisms of Tween20 and BSA further. In case of different modes of action but similar effects, these could act potentially additively or synergistically. However, it could also be that one additive simply surpasses the effect of the other. For example, if the interaction of Tween20 with the substrate is stronger than for the BSA, potentially the BSA will not interact with the substrate, but would be available as free protein in the solution without an actual function. Additionally, Eriksson et al. (2002) tested the combination of BSA and Tween20 and did not find a further increased conversion rate compared to scenarios with the individual additives.

Overall, the impact of the additives on the reaction varied between the tree species, an influence of bark was detected and species-specific effects indicated, which thus contribute to Rq 3.1 and 3.2 as well as 2.3.

5.16 Species-specific inhibitory substances

The previous experiments showed strong discrepancies between the enzymatic digestibility of the tree species especially between beech and oak or chestnut. The characterization of the materials showed that these tree species differ greatly in their chemical composition. Therefore, the supernatants of beech and oak db after 48 h of hydrolysis were comparatively examined by GC/MS analysis (chapter 4.2.5 and 4.3.5). Representative chromatograms of the GC/MS analyses for beech and oak db are included in the appendix (Figure 53, page 195). Three peaks could be detected, which distinguish oak db from beech db. The peak at a retention time of 19.1 min could not be identified by mass spectra interpretation. The peak at 20.7 min was aligned to the lignan lyoniresinol. This substance was also present in the LPS of oak (compare chapter 5.8.1). Additionally, ellagic acid was identified as most intense peak solely present in the supernatant of oak db.

As a typical component of oak and chestnut tannins and because of good availability of the purified substance, ellagic acid was chosen as a test substance. Following EH scenario 6 the SPS of beech db was staggered with ellagic acid (dry) in the respective concentration, which was found in the supernatant. The ellagic acid was quantified with 11.1 mg/l in the supernatant of oak, evaluated by an external calibration. Figure 35 presents the glucan conversion over 48 h in comparison to the control scenario (EH scenario 2). It was observed that ellagic acid as individual substance had no detectable effect on the glucan conversion of beech db. Similar results were reported by Tejirian and Xu (2011), where also no inhibitory effect of ellagic acid could be detected. However, probably the poor solubility of ellagic acid in aqueous solutions was one factor, which limited the interaction with the enzymes in the present experiments. Another explanation could be that the detected ellagic acid as individual substance had no effect, but within a bigger molecular structure (ellagitannins) it would probably have shown an effect on the enzymatic hydrolysis. Probably the original tannin structure dissolved in the supernatant of oak db was cleaved during the derivatization reaction or the gasification process and injection into the GC/MS device. The detected sugar peaks in the first part of the chromatogram (appendix, Figure 53, page 195) indicated the cleavage of glycosides, as saccharides were separated prior to the measurements by solid phase extraction. The presence of glycosides would agree with bigger tannin structures, due to the esterification of galloyl- units with glucose. Additionally, ellagic acid is the cleavage product of ellagitannins (Fengel & Wegener, 2003).

These analyses showed some differences regarding the dissolved substances within the supernatants after enzymatic hydrolysis. However, for the detected substance ellagic acid, no effect on the enzymatic digestibility could be observed. These results contributed to research question 3.1 and 3.2

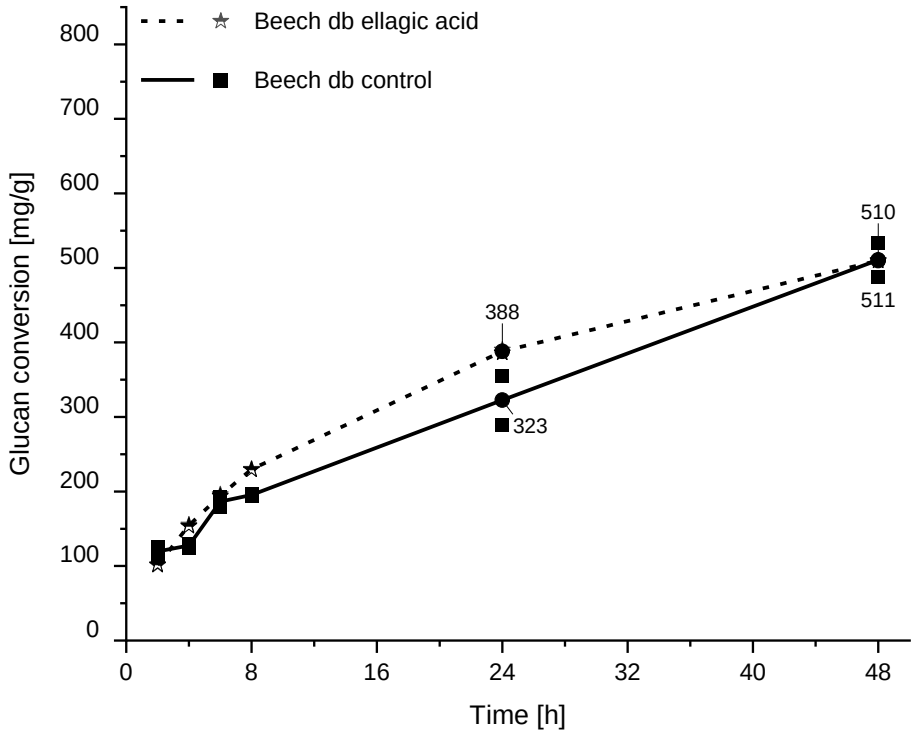


Figure 35: Enzymatic conversion of beech debarked (db) without (control) and with the addition of ellagic acid (ellagic acid); average values are represented by the lines and the symbols represent the individual measurements.

5.17 Effect of an intensified washing of the SPS prior to EH

Next to species-specific substances, residual potentially inhibitory substances, like furfural or hydroxymethylfurfural from the LPS could be included in the SPS due to insufficient separation of the process streams. Additionally, residual dissolved sugars could also led to inhibition during EH. For instance cellobiose could hamper CBHs and glucose could inhibit BGLs (Annamalai et al., 2016b; Ouyang & Xu, 2016) To investigate this aspect the SPS of chestnut and oak db was repeatedly washed in the laboratory with warm water (chapter 4.2.9), prior to enzymatic hydrolysis, following EH scenario 7. In Figure 36 the results for oak a) and chestnut b) db are presented by comparison of the glucan conversion of the control and the intensively washed material (washed). It was found that the double washing of oak db increased the glucan conversion about +18.5 % and for chestnut db about +30.6 %. Therefore, the additional washing process was an effective measure to increase the enzymatic conversion and points out the importance of a sufficient separation of the process streams. However, the glucan conversion rate of the intensively washed material did not reach the level of beech or ash.

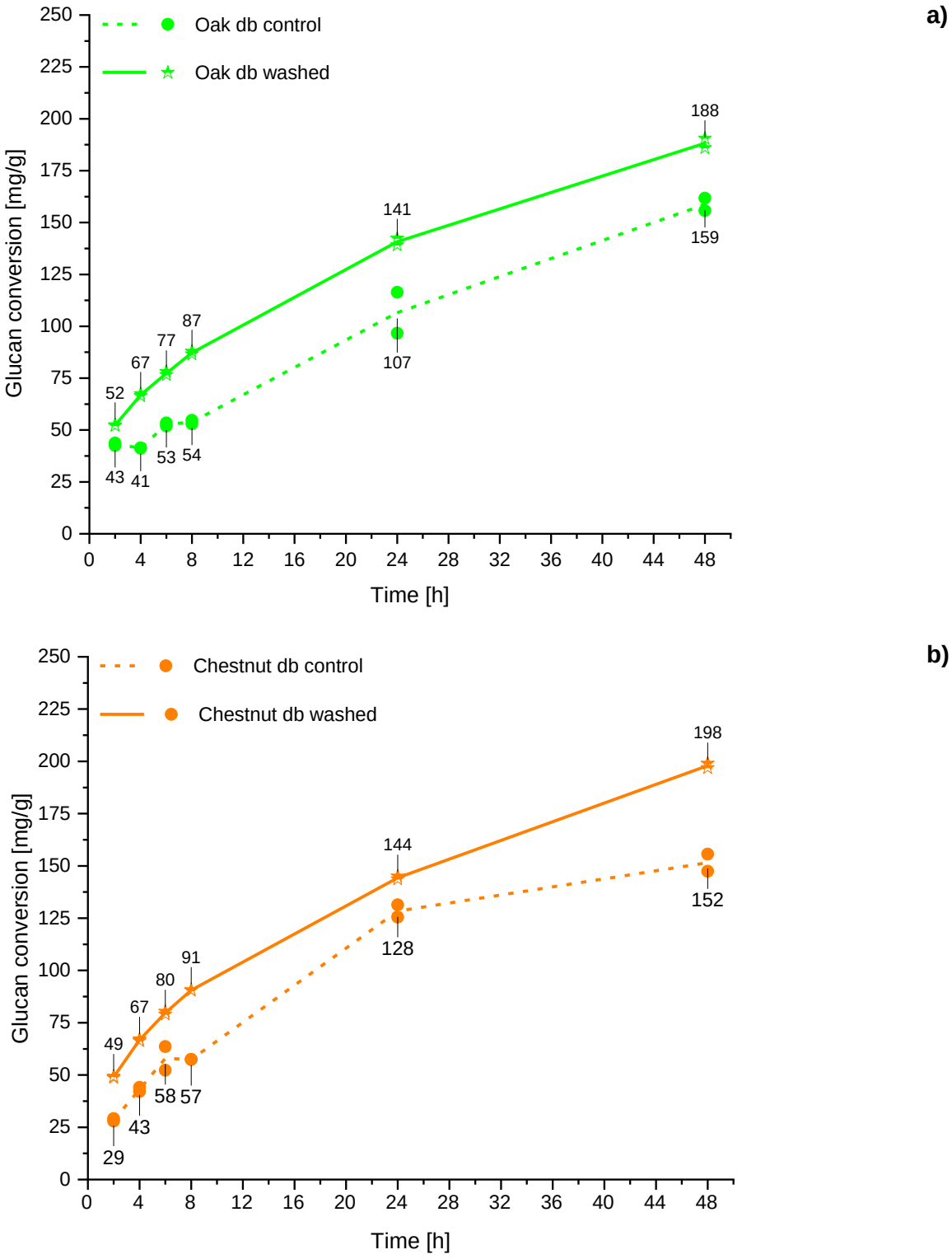


Figure 36: Glc conversion over time of oak a) and chestnut b) debarked (db) without (control) and with extra washing step (washed); average values are represented by the lines and the symbols represent the individual measurements.

Additionally, it was investigated, if the effect of the additional washing step in combination with the effect of Tween20 potentially could increase the conversion rate further (EH scenario 8).

The respective enzymatic conversion is presented in Figure 37. It was observed that the removal of the residual soluble fraction had no additional positive effect. Regarding these results it was assumed that Tween20 solely affects the non-soluble part of the sample. This would be in agreement with literature, which reported an interaction at the liquid-solid interface. Another hypothesis would be that the effect of Tween20 outraged the negative effect of the residual soluble substances, which would make an additional washing step theoretically redundant.

In regard to the research questions, these results contributed to Rq 3.1 and also to Rq 3.2 as these findings indicated that the strong hampering effect detected for oak and chestnut was also not related to the dissolved fraction, but to the insoluble solid fraction.

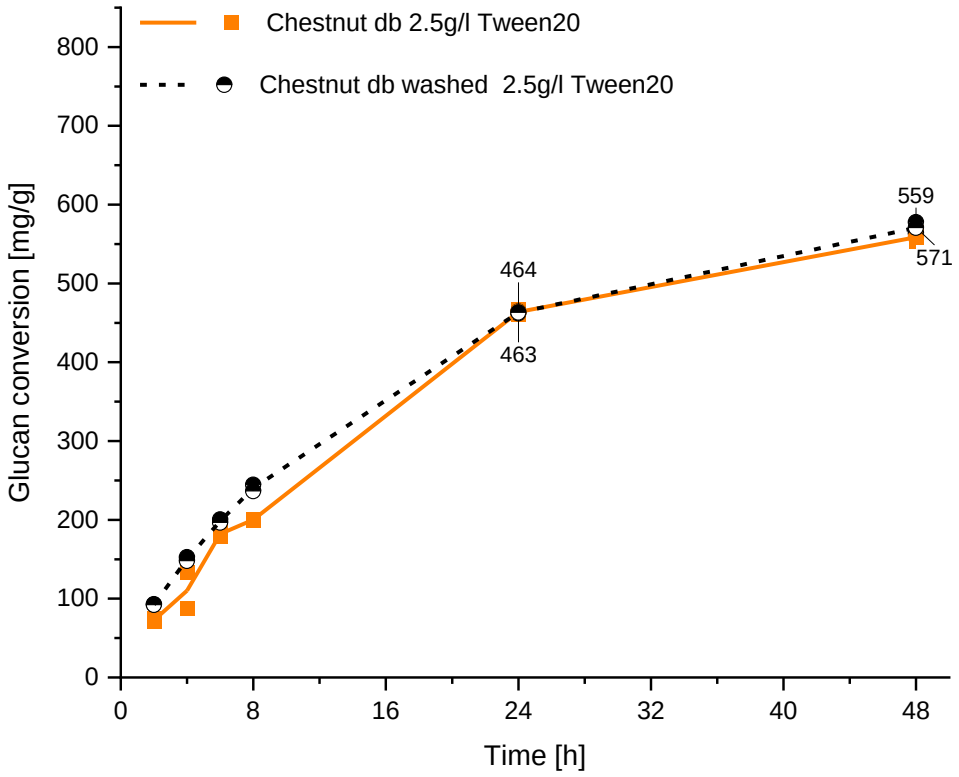


Figure 37: Glc conversion over time of chestnut debarked (db) with 2.5g/l Tween20 and double washed chestnut db with 2.5g/l Tween20; average values are represented by the lines and the symbols represent the individual measurements.

Part 3 Species-specific effects and approaches to improve the enzymatic digestibility

This third part concentrated on research questions (Rq) 3.2 and 3.3 but also touched Rq 3.1. The focus was set on the investigation of species-specific effects and the underling mechanisms. Additionally, different approaches to equalize species-specific effects during enzymatic hydrolysis were examined.

Figure 38 presents the centered research area and Rq as well as an overview on the included analyses.

3. The third research area dealt with different factors influencing enzymatic hydrolysis and the detectable variations related to tree species-specific effects.

3.1. *How to evaluate influencing factors in terms of impact on enzymatic hydrolysis?*

3.2. *How are the tree species-specific characteristics interconnected with influencing factors on enzymatic hydrolysis?*

3.3. *Which options are available to deal with potential species-specific effects during enzymatic hydrolysis?*

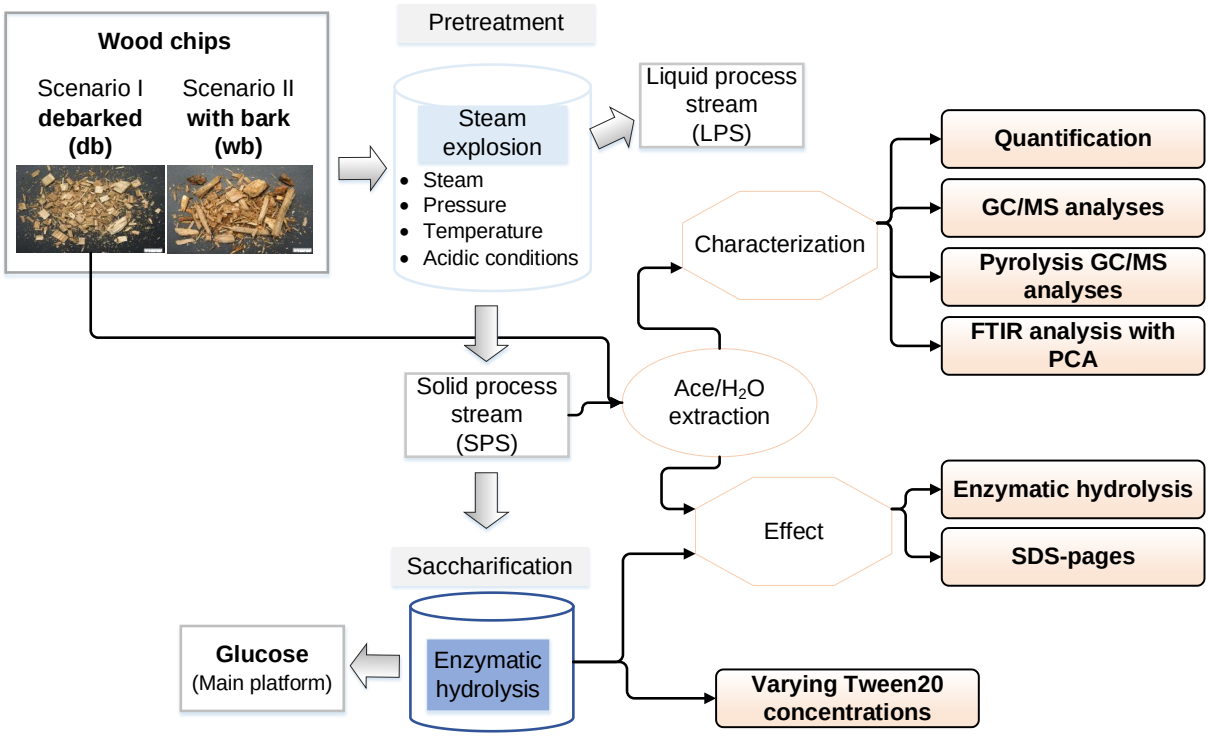


Figure 38: Overview on the analyses used to work on part 3: Species-specific effects and approaches to improve the enzymatic digestibility

5.18 The effect of different Tween20 concentrations

The results of the experiments regarding the non-productive binding effect showed a higher protein adsorption capacity for oak and chestnut. Additionally, it was shown that the additive Tween20 enhanced the enzymatic conversion strongly for these species and simultaneously reduced the enzyme adsorption. Therefore, one approach to equalize the species-specific effects was to test different Tween20 concentrations and their effect on the enzymatic hydrolysis. For this the concentration of Tween20 was halved (1.25 g/l) and doubled (5 g/l), which led to the EH scenarios 5 and 6 with scenario 3 as reference (see chapter 4.2.9). Both additional scenarios were applied for oak and chestnut db. The scenario with half of the original Tween20 concentration was added, to investigate if the chosen Tween20 concentration in EH scenario 3 was potentially too high, which would enable the same effect at lower Tween20 concentrations, but with lower material usage.

Figure 39 and Figure 40 present the glucan conversion of oak and chestnut db over time for the two different Tween20 concentrations and the reference scenario with 2.5 g/l. Interestingly, for both scenarios lower conversion rates were observed than for the reference concentration.

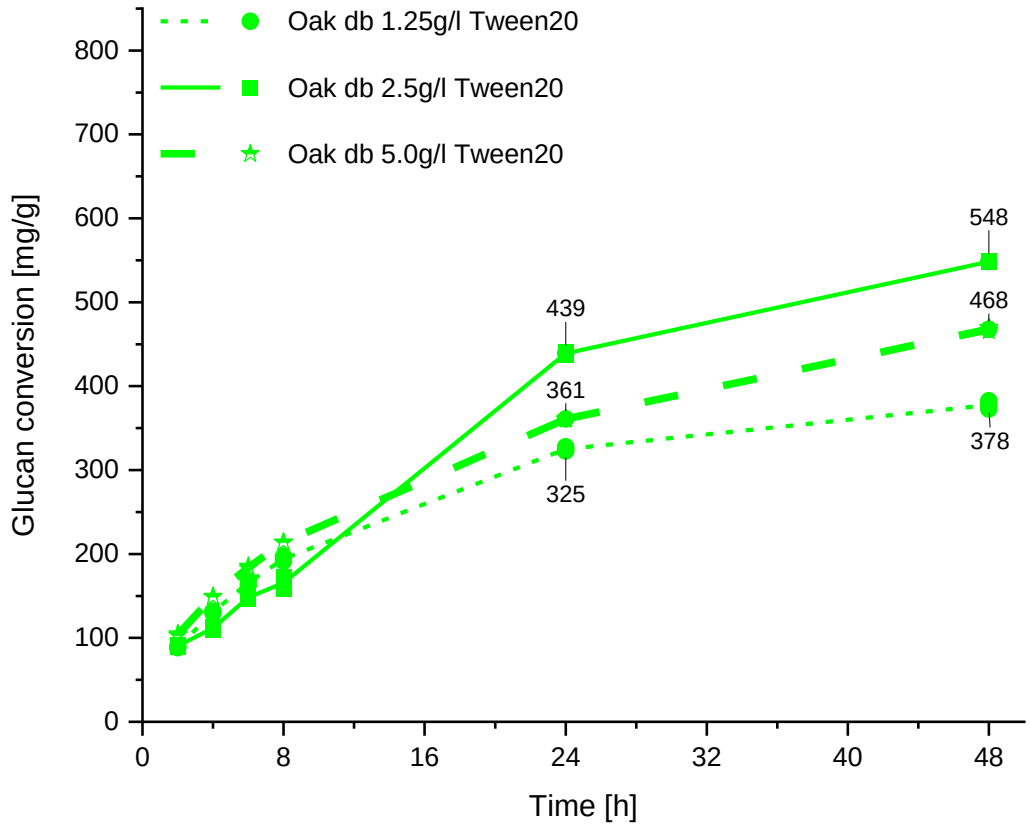


Figure 39: Enzymatic conversion of oak debarked (db) with different Tween20 concentrations; average values are represented by lines and the symbols represent the individual measurements

The halved Tween20 concentration showed conversion rates of 37.8 % for oak db and 38.3 % for chestnut db, which corresponded to a reduction of ~31 % in both cases. This indicated that the Tween20 concentration correlates with the impact strength. The reduced conversion rates, thus were reasonable, as the Tween20 concentration was probably too small to compensate the non-productive protein adsorption on the substrate in the same extent as at the higher concentration of 2.5 g/l.

For the scenario with 5.0 g/l Tween20 the conversion rate of oak was 46.8 % and for chestnut db it was 50.1 % after 48 h. These values were higher than for the scenario with 1.25 g/l, but still notably lower compared to the reference concentration with 2.5 g/l. This result was unexpected and the opposite of the statement in literature, where a nearly linear correlation was found for the hydrolysis conversion and the Tween20 concentration, with an increase from 2.5 to 5.0 g/l (Eriksson et al., 2002). In this case, the discrepancy to the values from literature could be possibly explained by the different proportions of Tween20 to the sample mass, which was 5 % in literature and in the present experiment it was about 12 %.

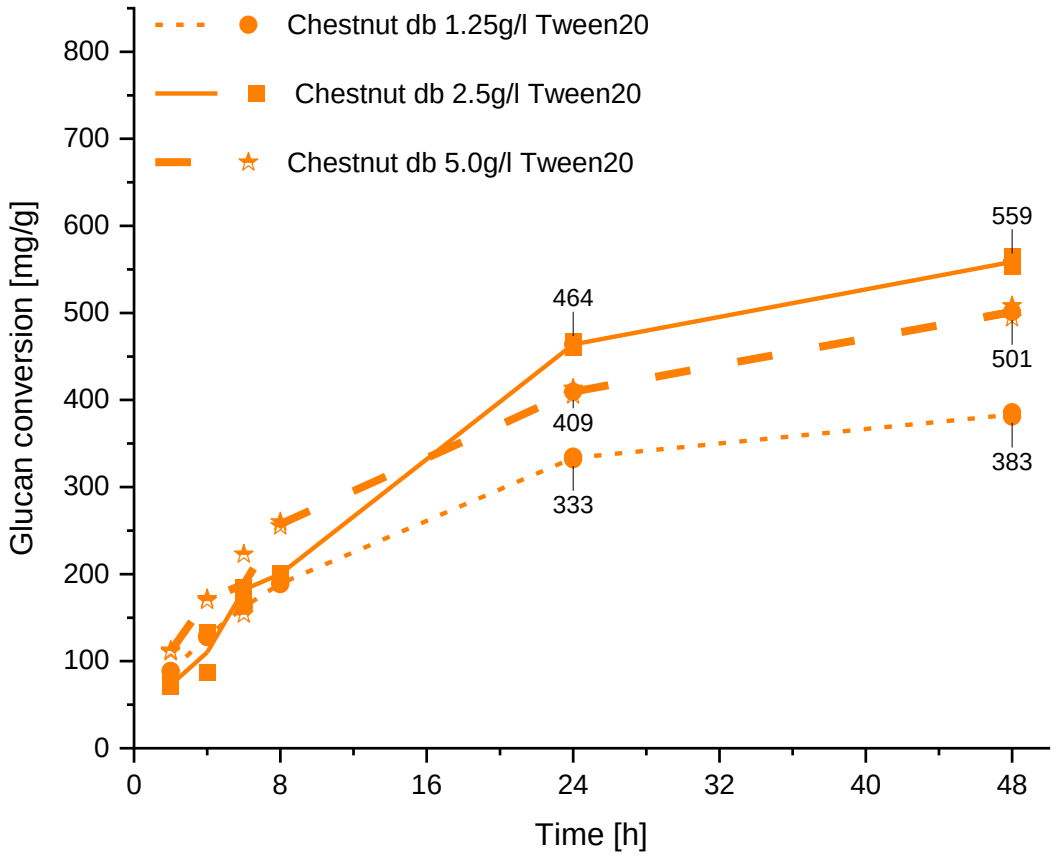


Figure 40: Enzymatic conversion of chestnut b) debarked (db) with different Tween20 concentrations; average values are represented by lines and the symbols represent the individual measurements.

A first hypothesis to explain the results was the assumption that at higher concentration the Tween20 molecules form micelles. The formation of micelles could possibly reduce the interaction of Tween20 with lignin, due to an orderly structural arrangement of the molecules. However, the critical micelle concentration of Tween20 was reported with 0.059 mM (Sivars & Tjerneld, 2000). This corresponds to a Tween20 concentration of ~0.072 g/l and thus even at a concentration of 1.25 g/l micelles were formed. The forming of micelles thus seemed not to influence the effect of Tween20 in relation to enzyme adsorption. This is in agreement with findings from literature as Tween20 was adsorbed as monomers to the lignin surface and not as micelles (Seo et al., 2011).

Tween20 interacts with lignin over hydrogen bonds, which are stronger in lignin than in polysaccharides due to the aromatic structures in lignin. These interactions were used to explain the higher affinity of Tween20 towards lignin than to polysaccharides (Kaiser, 2018). However, it could be assumed that at a specific concentration the molecules start to interact with the cellulose surfaces by hydrogen bonding as well, because the respective sites at the

lignin were already occupied. This hypothesis could explain a reduced glucan conversion, as the enzymes were unable to attach to the cellulose surface due to a shielding effect.

Another hypothesis could be that the hydrophobic parts of the Tween20 molecules start to interact with the enzymes in solution as well, which could create a spatial hindrance effect. Zhou et al. (2015) for instance observed a reduced cellulose conversion during enzymatic hydrolysis of pure cellulose at high Tween20 concentrations. This was explained by interactions of the Tween20 with cellobiohydrolases (CBHs). This decreased the productive adsorption of the enzymes to the cellulose surface. Parallel an increased proportion of protein in the solution was detected (Zhou et al., 2015). Overall, there were some approaches available to explain the observed results, but the mechanism could not be resolved completely within the here performed analyses.

In regard to Rq 3.2 as well as partly Rq 3.3 it was shown that the prevention of the non-productive adsorption effect was dependent on the Tween20 concentration, but could not explain the species-specific effect of oak and chestnut completely and the positive effect could not be increased further.

5.19 Ace/H_2O extraction of wood and the SPS

Based on the previous analyses and results two assumptions were made:

1. The main hampering effect of oak and chestnut SPS was part of the water-insoluble fraction of the material.
2. The species-specific effect detected for oak and chestnut was not exclusively based on the non-productive binding of enzymes to the substrate but was probably due to further effects like absorption and inactivation of enzymes.

Therefore, the processed experiments and the respective results within the following chapters were developed from these assumptions.

It is commonly known that hydrolysable tannins interact with proteins, but it is not completely revealed how these tannins react during the steam explosion process and how this affects the material properties and following processes like enzymatic hydrolysis. However, some researchers detected or assumed a condensation of tannins during steam explosion (Boussaid et al., 2001; Lomax et al., 1994). This would agree with the assumption that the main effect accounts to the insoluble substrate proportion. However, these studies were performed on softwoods, which contain mainly condensed tannins. Hydrolyzed tannins are linked by ester linkages or C-C (McManus et al., 1985). Therefore, it was assumed that these molecules potentially undergo depolymerization and repolymerization and condensation reactions as well. Next to the tannins, changes in the lignin structure or the formation of so called “pseudo-lignin” or “humin-like” substances, which contain polymerized sugar degradation products (Aarum et al., 2018; Li et al., 2009) should be considered as well to discuss the detected effects.

To get deeper insights in that topic the focus was set upon an attempt to extract a potentially tannin rich fraction from the material. For this an extraction with acetone and water (Ace/H_2O) was chosen. The method was based on the gentle extraction method used for technical tannins by Streit (1993). To evaluate the changes, which happen during the SE process similar extractions were performed on the wood and the SPS materials. To enable a better observation of changes related to tannins, material from oak db and chestnut db and as a reference from beech db were extracted, because beech is known to contain no hydrolysable tannins.

5.19.1 Proportion of Ace/H_2O extractives in wood and SPS

In a first step the native wood as well as dried SPS of beech, oak and chestnut db were successively extracted with petrol ether to remove fats and waxes followed by the extraction with Ace/H_2O. The petrol ether extraction yielded extract volumes < 0.2 % for all samples and thus was not considered further. Figure 41 shows the quantitative results of the Ace/H_2O extraction step of wood and the SPS for the debarked samples of beech, oak and chestnut. Focusing on the **wood**, the results clearly showed the lowest extract content for beech with 1.9 % followed by oak with 12.5 % and chestnut with 14.9 %. The higher extract content detected for chestnut db was in line with the results of the prior described successive extraction of wood (chapter 5.7). In this previous analysis the extract content of chestnut db for the acetone fraction was also higher than for oak db. This was explained by the difference of the trees age, which potentially led to a lower polymerization degree of secondary extractives (tannins) and thus were easier to dissolve within the younger trees (chestnut) (Klumpers et al., 1994).

For the **SPS** the extract content was notable higher than for wood, but this time oak showed the highest values with 25.9 % and beech showed the lowest values with 16.7 %. This showed that the acidic steam explosion increased the proportion of Ace/H_2O soluble materials in the SPS compared to wood. The change in ratio between the extract content of oak and chestnut is possibly reasoned in the prior discussed higher solubility of less polymerized extractives in the wood of chestnut. These extractives potentially were already dissolved during the steam explosion process and were transferred in the LPS and are thus not available in the SPS.

For the Ace/H_2O extraction of technical tannins and heartwood of *Quebracho colorado* Streit (1993) received a solubility of 95 % and 32 %, which represent much higher proportions than detected here. This tree species is known to contain a high proportion of condensed tannins. In Wagenführ (1984) a tannin content for *Quercus robur/petraea* of 3 - 13 % in heartwood and just 1 % in sapwood was reported. Additionally, for *Castanea sativa* a tannin content of 7 - 16 % in 60 to 80 year old trees or 8 % for *Eucalyptus diversicolor* was given. These examples point out the high variability in tannin content detectable in the same species. However, the determined values for oak and chestnut wood agreed well with the ranges given in literature.

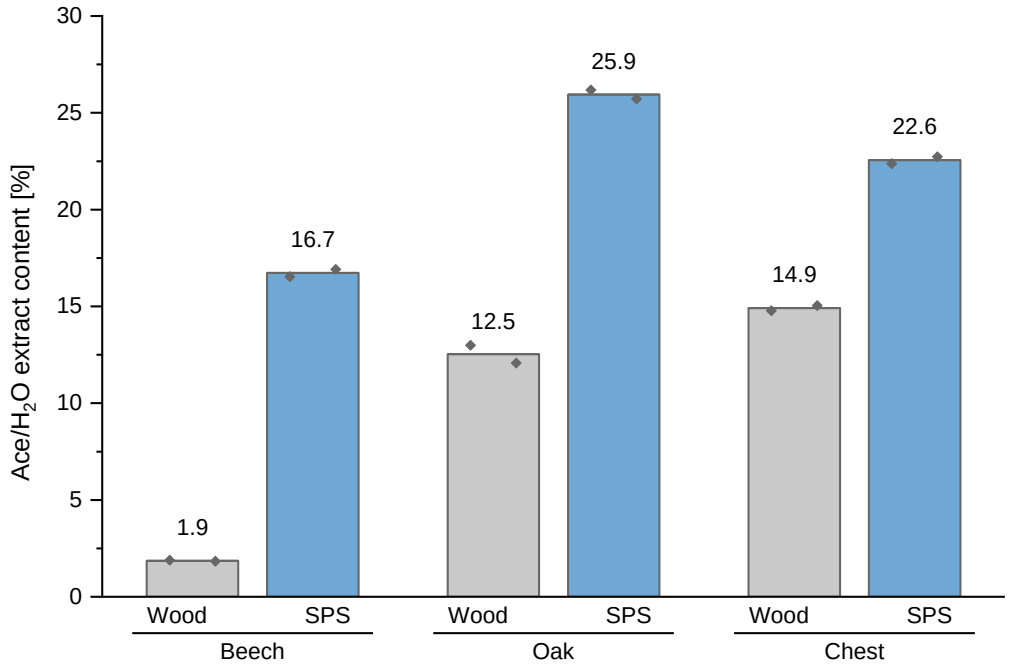


Figure 41: Ace/H_2O extract content of wood and SPS of beech, oak and chestnut.

5.19.2 Characterization of the Ace/H_2O extracts by FTIR and Principal Component Analysis (PCA)

The Ace/H_2O soluble fraction was first characterized by FTIR measurements to picture the whole sample. Further, the spectra were evaluated by Principle Component Analysis (PCA).

Differences between the Ace/H_2O extracts from wood

Figure 42 presents the fingerprint region (1800 – 700 cm^{-1}) of the FTIR spectra of the Ace/H_2O extract originated from **wood** respectively for oak, chestnut and beech (average spectra of 6 - 12 measurements).

To start with, first the spectra of the **Ace/H_2O extracts** from **wood** were shortly compared with the spectra of the **hot water extracts** from **wood** (chapter 5.7.4, Figure 13). It was observed that the spectra for oak and chestnut looked similar, but for beech some differences could be detected. In the spectra of the Ace/H_2O extract of beech the signal around 1500 cm^{-1} is sharper as well as the signals at 1450 cm^{-1} and around 1200 cm^{-1}. Additionally, the signal at 1120 cm^{-1} is stronger and sharper than in the hot water extract. These signals indicate aromatic skeletal vibrations and CH_2 scissoring or OH plane deformation as well as C-O-C asymmetric valence vibration assigned to cellulose (Salmén & Bergström, 2009; Schwanninger et al., 2004). Overall, these findings showed that the extracted fractions differ for beech, but for oak and chestnut both solvents solved similar substances from the wood. It is known that tannins are industrially extracted from wood by solvents like hot water, next to others (Mai & Zhang, 2023). The previous presented GC/MS analyses of the hot water extracts revealed proportions of gallic and ellagic acid (chapter 5.7.2), which are typical components of hydrolysable tannins. Additionally, the signals of the FTIR spectra of the hot water extract of oak showed signals characteristic for hydrolysable tannins. Therefore, the similarities between the spectra for oak and chestnut for both extractions were a first indication that the Ace/H_2O extract from wood also contained tannins.

By comparing the spectra of wood **Ace/H_2O extracts between the species** (Figure 42) it became obvious that oak and chestnut showed similar patterns, but strong differences could be observed for the spectrum of beech.

The absorbance band around 1740 cm^{-1} in the spectra (Figure 42), which is dominant in oak/chestnut but minor in beech can be assigned to different functional groups. Signals in this region could be assigned to C=O valence vibrations of acetyl- or carboxyl groups. Further, it could be signals of C=O stretch, which are attributed to unconjugated ketons, carbonyl groups and ester groups (Schwanninger et al., 2004). Additionally, during analyses of pure ellagic acid the IR signal at 1725 cm^{-1} was assigned to C=O stretching in the ester groups (Goriparti et al., 2013) (support information) and Liu et al. (2021) attributed this signals around 1740 cm^{-1} to esterified galacturonic acids.

The shift in the aromatic bands 1614 → 1605 cm^{-1} and 1514 → 1504 cm^{-1} of oak/chestnut and beech showed differences within the lignin structure (Özparpucu et al., 2018; Schwanninger et al., 2004).

To address the differences in the spectra between 1450 – 1400 cm^{-1} the signals are assigned as followed: The signal around 1450 cm^{-1} could be attributed to scissoring of CH_2 on pyran rings and OH deformation (Schwanninger et al., 2004), which was commonly related to carbohydrates, but can be influenced by lignin as well (Åkerholm & Salmén, 2001; Gierlinger et al., 2008). Therefore, the slight shift of the signal peak at 1450 cm^{-1} potentially indicated differences in the polysaccharide composition of the extracted fractions.

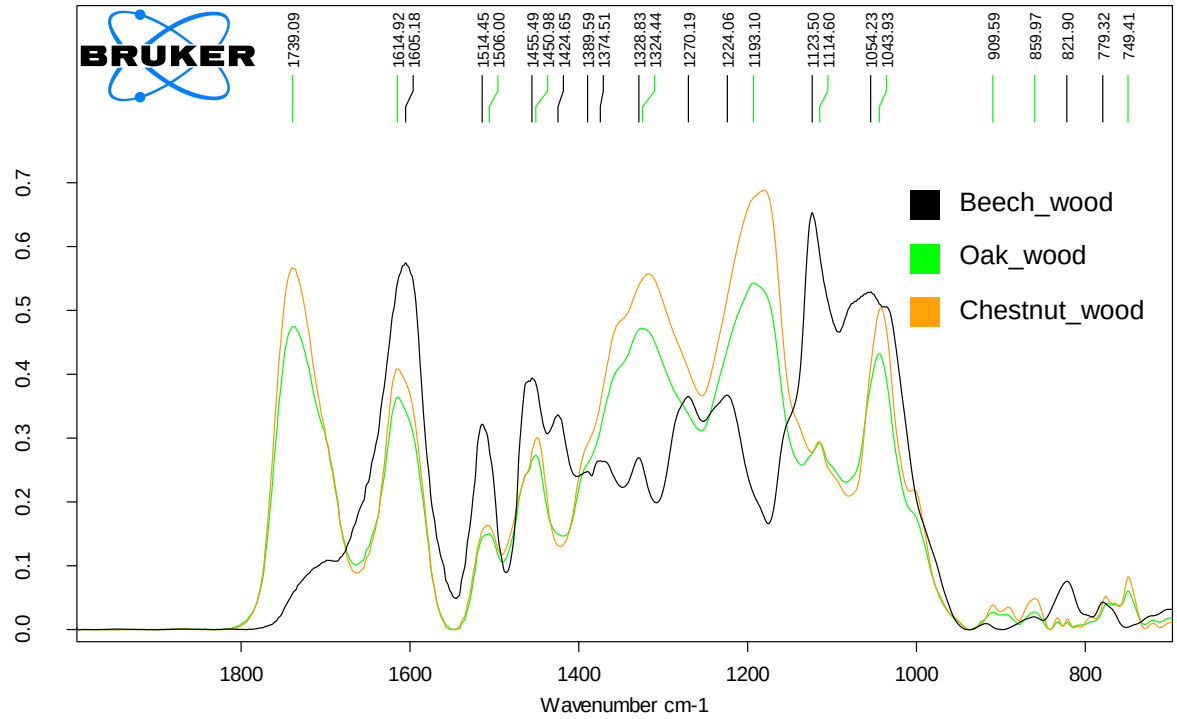


Figure 42: Fingerprint region of the FTIR spectra (baseline corrected) of the Ace/H_2O extracts originated from wood for oak, chestnut and beech; signal peaks are marked solely for the spectrum of oak and beech.

Additionally, the spectra of beech showed a signal around 1420 cm^{-1}, which is missing in the oak/chestnut spectra. The region between 1430 – 1422 cm^{-1} was assigned to aromatic vibrations combined with C-H deformations (Faix, 1991; Schwanninger et al., 2004). However, the signal at 1430 cm^{-1} can also be attributed to CH_2 scissoring in cellulose (Åkerholm & Salmén, 2001). Kačuráková et al. (1998) also assigned the regions between 1459 – 1447 cm^{-1} and 1428 – 1407 cm^{-1} to CH bending within hemicelluloses. The general presence of hemicelluloses in the extracts is likely as these sugars get more easily solubilized than cellulose due to their lower polymerization degree (Fengel & Wegener, 2003) and during the GC/MS analysis of the extracts some sugars were detected as well (chapter 5.19.3).Therefore, this could indicate a probably higher polysaccharide content in the Ace/H_2O extract of beech wood or that the proportions of lignins differ strongly between the species.

Further, oak and chestnut showed a wide signal around 1324 cm^{-1} and 1190 cm^{-1} (Figure 42). According to literature, signals around 1320 cm^{-1} were attributed to vibrations of phenolic OH groups. Signals at 1335 cm^{-1} could also be assigned to OH in plane bending vibrations in cellulose or syringyl ring breathing (Fengel & Wegener, 2003; Gierlinger et al., 2008; Schwanninger et al., 2004). The signal at 1190 cm^{-1} was assigned to the vibration of ester bonds in ellagic acid (Goriparti et al., 2013) (support information). In contrast, beech showed just a small peak at 1328 cm^{-1} and additionally a peak at 1270 cm^{-1}, which is assigned to G- lignin. Interestingly, this signal was not observed in the spectra of oak and chestnut or was probably covered by the wide signal around 1324 cm^{-1}.

Beech showed a sharp peak at 1120 cm^{-1} compared to oak and chestnut. In contrast, around 1040 cm^{-1} beech showed a wide signal, but oak and chestnut showed a sharp intense absorbance band. Goriparti et al. (2013) assigned the signal of pure ellagic acid at 1052 cm^{-1} to ester bonds, which could potentially correspond to the 1040 cm^{-1} signal determined here. This signal thus agreed to the signals at 1190 and 1740 cm^{-1}, which were also assigned to esters (Goriparti et al., 2013). Additionally, the signals between 1120 cm^{-1} and 1107 cm^{-1} were attributed to ring stretching and vibration as well as C-O, C-C stretching (Schwanninger et al.,

2004), which is commonly corresponded with sugars. This supported the assumption of higher cellulose or polysaccharide contents in the extract of beech.

Overall, the signals between 1731 – 1704 and 1325 – 1317 cm^{-1} were reported as characteristic bands for hydrolysable tannins (Falcão & Araújo, 2013). Therefore, in combination with the individual signal assignments it was strongly indicated that hydrolysable tannins were present in the Ace/H_2O extracts of oak and chestnut wood. However, these signals are potentially partly overlapping with sugar related signals.

The wood Ace/H_2O extracts were additionally evaluated by PCA, applied after min/max normalization and 1st derivatization of the spectra (Figure 43). PC-1 explained 98 % of the variability and separated the extract of beech from oak and chestnut wood. The first loading indicated the bands around 1760, 1590, 1500, 1470 cm^{-1} as well as 1290, 1160 and 1130 cm^{-1} as important signals to explain the variations. These loadings refer to the differences already described by visual comparison and thus did not extract further information.

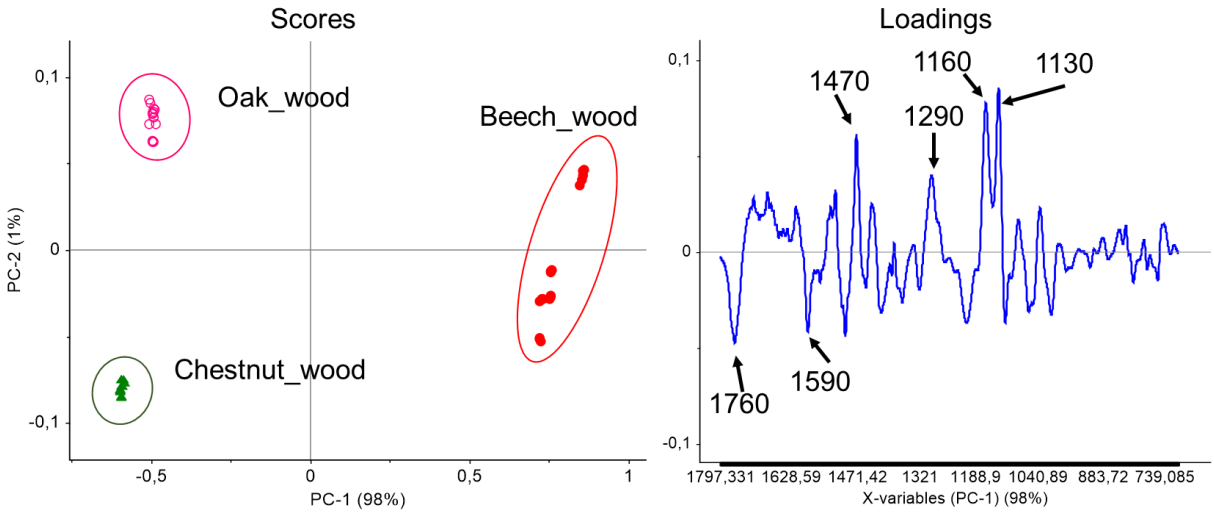


Figure 43: PCA scores of the Ace/H2O extracts from wood (left) and the respective loadings (right); spectra were min/max normalized and 1st derivatization was applied.

Differences in the Ace/H_2O extracts from wood and the SPS

The spectra (fingerprint region) of wood and the SPS for beech and oak, representing also chestnut, are presented in Figure 44 a) and b) (average spectra from 6 - 12 measurements).

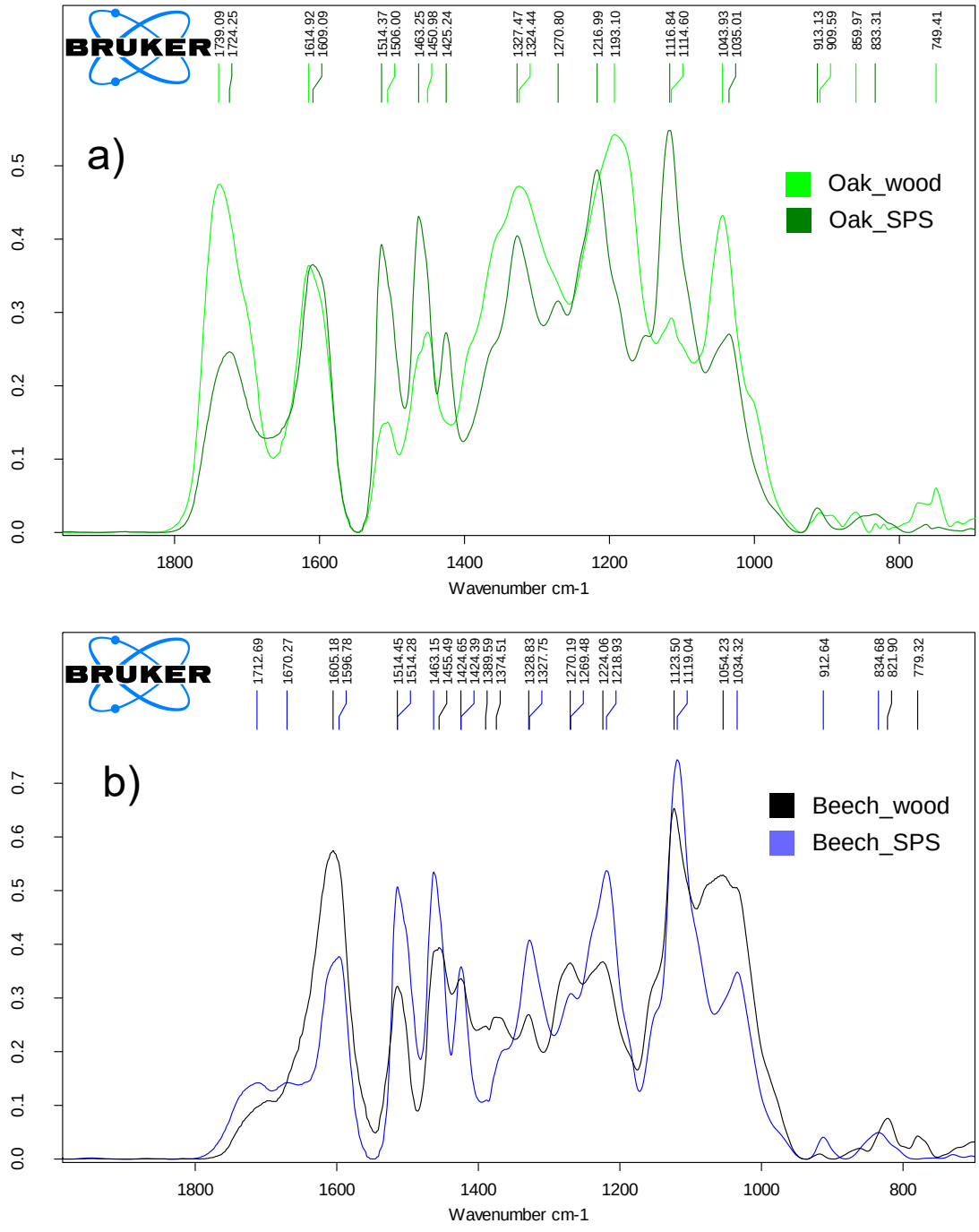


Figure 44: Fingerprint region of FTIR spectra (baseline corrected) of a) oak wood and SPS and b) beech wood and SPS; signal peaks are marked solely for the spectrum of oak and beech.

Comparing the spectra of the wood extracts with the SPS extracts several major changes appear.

For **oak** (also as reference for chestnut) the ratio between the signal around 1740 and 1600 cm^{-1} changed. Additionally the intensity of the peak at 1320 cm^{-1}, which was described as characteristic signal for hydrolysable tannins like the signal at 1730 cm^{-1} decreased (Falcão & Araújo, 2013). However, signals around 1330 cm^{-1} could also be attributed to syringyl units (Fengel & Wegener, 2003). Further, there is a shift of the signal peak at 1190 cm^{-1} in the extract to wood towards 1216 cm^{-1} in the SPS extract. Whereas, the signal at 1190 cm^{-1} was attributed to ester bonds (Goriparti et al., 2013), signals around 1200 cm^{-1} were attributed to OH in plane bending and C-O-C stretch in cellulose or to C-C and C-O at 1227 cm^{-1} in lignin of beech (Gierlinger et al., 2008). The signal around 1040 cm^{-1} in the extract of wood also decreased and the peak shifted to 1035 cm^{-1}. In the wood extract, this signal could be possibly attributed to ester bonds in ellagic acid or C-C and C-O ring signals in xyloglucans or C-O valance vibration of cellulose. Signals around 1035 cm^{-1} are attributed to C-O valence vibrations or C - O stretch in cellulose as well as aromatic C-H in plane deformation plus C=O stretch, which would be typical for S-lignin units (Gierlinger et al., 2008; Goriparti et al., 2013).

This changes led to the assumption that potentially on the one side, the proportions of chemical structures commonly associated with hydrolysable tannins, like ester bonds (1730, 1190, 1040 cm^{-1}) decreased in the SPS extracts of oak compared to the extracts from wood. On the other side it could be probably assumed that the composition of aromatics and sugars also changed.

This assumption is supported by the following observations: in the spectra of the SPS extract of oak a signal at 1270 cm^{-1} attributed to G-lignin becomes visible. Further, the aromatic signal around 1500 cm^{-1} (Schwanninger et al., 2004) became more intense and sharper and the signals shifted slightly to higher wavenumbers. Further, at 1420 cm^{-1} an additional peak appeared, which can be assigned to aromatic vibrations combined with C-H deformations (Faix, 1991; Schwanninger et al., 2004). Therefore, it could be assumed that the signals associated with the aromatic compounds became more intense and sharper, which potentially indicates a change in the structure of these aromatic compounds and a probably higher degree of polymerization (signals at 1500 and 1430 cm^{-1}). Özparpucu et al. (2018) also assigned shifts in the absorption bands related to lignin with changes in the lignin composition of genetically modified wood samples. Further, Li et al. (2009) also reported higher condensed lignin structures due to repolymerization of lignin during SE.

It has to be considered that signals around 1740 and 1420 cm^{-1} also can be associated with hemicelluloses or esterified uronic acids (Kačuráková et al., 1998; Liu et al., 2021). Therefore, a decreased signal intensity could also be reasoned in a reduced amount of hemicelluloses and the corresponding acetyl groups (Gierlinger et al., 2008), as these got dissolved during the SE process. This could be an explanation for the decreased signal intensity at 1740 cm^{-1}, but the signal around 1420 cm^{-1} appeared solely in the SPS extract and was not present in the wood extract.

Additionally, in the spectra of oak SPS extracts the signal around 1450 cm^{-1}, which is attributed to scissoring of CH_2 in pyran rings and OH deformation (Schwanninger et al., 2004) became more intense and sharp. The intensity of the signal around 1115 cm^{-1} is notably increased as well. This signal is attributed to cellulose in the region of 1115 cm^{-1} and to xyloglucan around 1118 cm^{-1}. However, in lignin samples from beech the signal at 1126 cm^{-1} was attributed to aromatic C- H in plane deformation plus C=O stretching, which is typical for S-lignin units (Gierlinger et al., 2008). These changes supported the assumption of differences within the sugar related composition between extracts originated from wood and the SPS.

For **beech** the ratio between the signal around 1700 and 1600 cm^{-1} changed as well and the signals between 1500 – 1400 cm^{-1} got sharper and more intense. Further, the signal at 1330 and 1220 cm^{-1} became more intense and the wide signal around 1050 cm^{-1} in the wood extract decreased and shifted to 1030 cm^{-1}. However, in beech extracts the signal at 1330 cm^{-1} was likely not characteristic for hydrolysable tannins, but probably attributed to syringyl-units (Fengel & Wegener, 2003). Therefore, similar to oak/chestnut some differences in the chemical structure of aromatics and sugars could be assumed for beech as well.

For revealing if there are any invisible differences another PCA was performed to compare the Ace/H_2O extracts from wood and the SPS for the respective species. The scores and loading (PC-1) are presented in Figure 45. PC-1 explained 84 % of the differences and separated the extracts from wood from the extracts originated from the SPS. The strongest signals in PC-1 were around 1520, 1470, 1430 cm^{-1} as well as around 1200, 1160 and 1130 cm^{-1}, with most signals already described in detail in the prior paragraphs. Therefore, in this case the PCA did not reveal additional differences between the samples, but supported the visually detected differences.

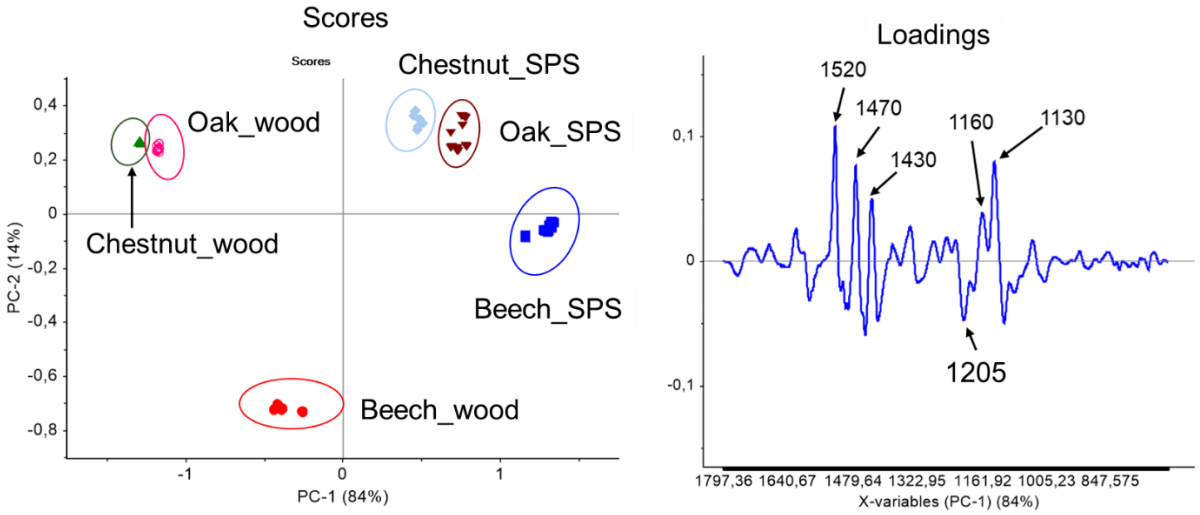


Figure 45: PCA scores of the Ace/H_2O extracts of wood and the SPS of beech, oak and chestnut (left) and the respective loading (right); spectra were min/max normalized and 1st derivatization was applied.

Differences between the Ace/H_2O extracts from the solid process stream (SPS)

Figure 46 presents the fingerprint region (1800 – 700 cm^{-1}) of the FTIR spectra of the Ace/H_2O extracts originated from the solid process stream (SPS), respectively for oak, chestnut and beech (average spectra of 6 - 12 measurements). Interestingly, the spectra of the species now appear more similar. Considering the presented changes between the extracts from wood and SPS as well as previous analyses (chapter 5.8.2) it was shown that the SE process influenced the composition and proportions of dissolvable substances notably.

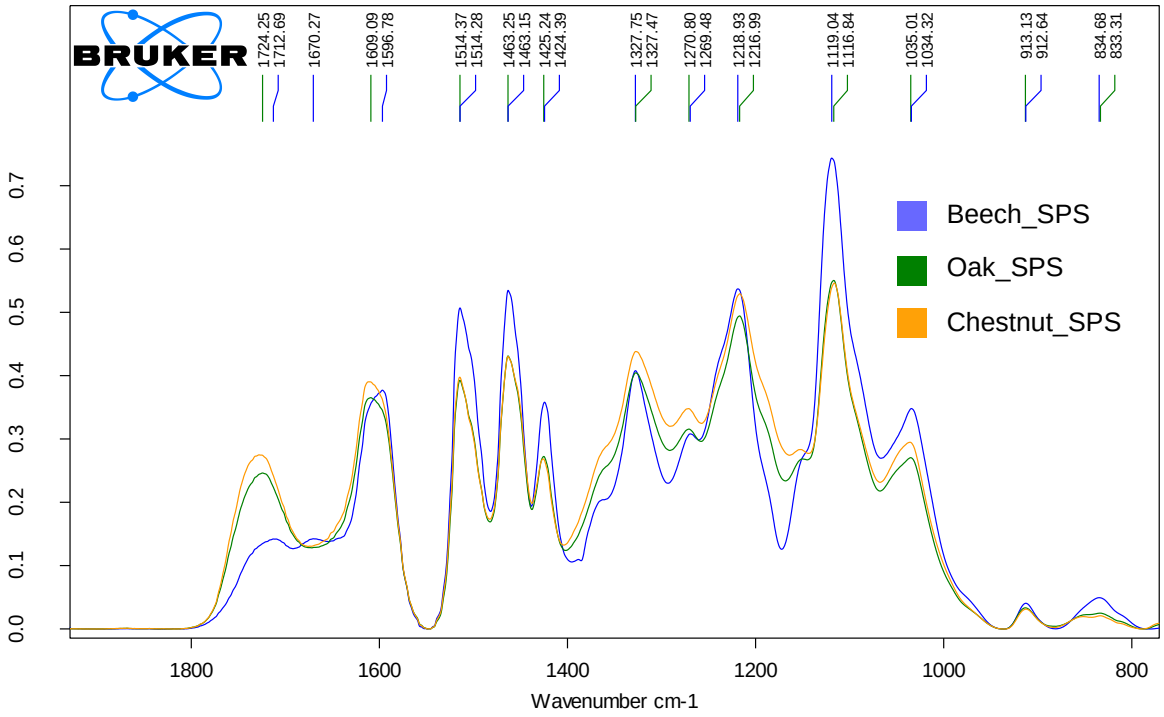


Figure 46: Finger print region of FTIR spectra (baseline corrected) of the Ace/H_2O extracts originated from the solid process stream, respectively for oak, chestnut and beech; signal peaks are marked solely for the spectrum of oak and beech.

Regarding the differences between the SPS extracts of beech and oak/chestnut the main signal, clearly separating the spectra is the signal around 1740 cm^{-1}, which shows a notable absorbance for oak/chestnut, but for beech it was more like a plateau next to the peak at 1600 cm^{-1}. The signal around 1740 cm^{-1} was described as a typical signal for hydrolysable tannins and can be attributed to ester bonds. However, also sugars or esterified uronic acids can contribute to this signal (Falcão & Araújo, 2013; Goriparti et al., 2013; Liu et al., 2021).

The aromatic signals around 1600 cm^{-1} (Schwanninger et al., 2004) differ in their peak position, which possibly indicated differences in the aromatic compounds extracted from beech and oak/chestnut (Özparpucu et al., 2018).

Further, between the signals at 1216 and 1119 cm^{-1} in Figure 46 there is a small absorption band detectable in the spectra of oak and chestnut at 1150 cm^{-1}, which can be assigned to C – O - C asymmetric stretching in cellulose or xyloglucan (Åkerholm & Salmén, 2001; Gierlinger et al., 2008). This signal is just a slight shoulder in the spectrum of beech.

A PCA was performed for the Ace/H_2O extraction of the SPS. PCA scores and loading are presented in Figure 47. PC-1 explains 95 % of the variability and separates beech SPS extracts from oak and chestnut SPS extracts. The first loading showed a band around 1760 cm^{-1}, and around 1520, 1470 and 1430 cm^{-1} as well as 1200, 1160 and 1130 cm^{-1}. These signals refer to the already discussed changes, but supported the assumption of differences within the composition of aromatic and sugar related structures in the extracts of beech and oak/chestnut.

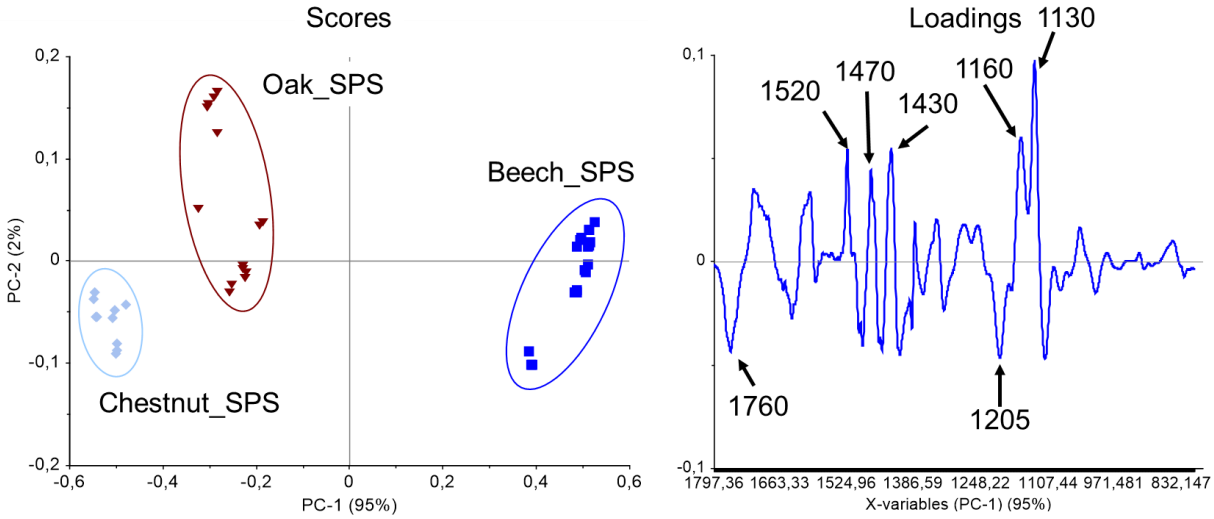


Figure 47: PCA scores of the Ace/H_2O extracts from the solid process stream (left) and the respective loadings (right); spectra were min/max normalized and 1st derivatization was applied.

The formation of humin-like substances ("pseudo-lignin") through the polymerization of sugar degradation products is known for industrial conditions during wood hydrolysis as well as steam explosion and other processes (Aarum et al., 2018; Sumerskii et al., 2010; van Zandvoort et al., 2013). Therefore, the potential presence of these substances within the extracts has to be considered as well. Experiments about the solubility of humin-like substances originated from monosaccharides by acid treatment showed a solubility < 20 % within acetone (Sumerskii et al., 2010).

Regarding FTIR analyses of humin-like substances, Sumerskii et al. (2010) assigned some absorption bands for humins at 1710 - 1685 cm^{-1} to furan derivatives, ester and aromatic and aliphatic compounds and the region between 1610 – 1560 cm^{-1} to furan rings. Further, the region 1440 - 1415 cm^{-1} was assigned to CH, -OH and COO^- and the region 1200 - 1000 cm^{-1} was attributed to ethers, acetals, ketals and furan rings. Pin et al. (2014) also gave some assignments of FTIR signals for humins, with 1020 cm^{-1} assigned to C-O stretch, 1600 cm^{-1} to C=O stretching conjugated with C=O or 1665 cm^{-1} with C=O from aldehydes, next to others. Considering these assortments the absorbance bands around 1600 and 1200 and 1119 cm^{-1} in the FTIR spectra (Figure 46) can be possible attributed to humin-like substances.

In a study from Hu et al. (2013) the differences between pseudo-lignin and dilute acid pretreated lignin were investigated by FTIR. The pseudo-lignin was prepared by acid treatment of holocellulose from *Populus trichocarpa x deltoides*. The FTIR band assignments of the pseudo-lignin were comparable with the assignments for humin-like substances of the previous mentioned studies. However, an intense band around 1697 cm^{-1} was detected for the pseudo-lignin and just a plateau for the acidic pretreated lignin, which were assigned to C=O signals in carbonyl or carboxyl groups. Compared to the presented results, here the Ace/H_2O

extractive spectra of beech (SPS), showed a slight peak around 1670 cm^{-1}, which was not present in the spectra of oak or chestnut (Figure 46).

Overall, the analyses of the FTIR spectra revealed a similar chemical structure of the extracts from oak and chestnut, but strong differences to beech. Further, the Ace/H_2O extracts from wood differed from the SPS extracts and a change in the structure of aromatic compounds as well as for tannin-related structures in oak and chestnut was potentially indicated. Further, there was no clear evidence for the presence or absence of humin like substances in the FTIR spectra of the SPS and potentially a mixture of lignin, humin and tannin related condensation products was present. However, these analyses need some supportive analyses to clarify the composition of the extracts further. Therefore, these results should be interconnected to the analyses in chapter 5.19.3.

5.19.3 Characterization by (pyrolysis) GC/MS

Next to the FTIR measurements the Ace/H_2O soluble fraction was characterized at GC/MS after derivatization as well as by pyrolysis GC/MS and by combination of pyrolysis GC/MS with on line silylation (see chapter 4.2.3, Ace/H_2O extraction).

GC/MS analyses after sample derivatization

For **beech** in both extracts from wood and the SPS, sugar related peaks had the major proportion in the chromatograms.

In the **wood** extract a not clearly identified organic acid and the flavonoid catechin were found next to the sugar peaks. Catechin had a proportion of 10.5 % in relation to the total extract proportion and based on the non-extracted material the proportion was 0.2 %. In contrast, in the **SPS** of beech other substances like the aldehyde form of syringol the syringaldehyde or syringaresinol a lignan were detected, next to sugar related peaks. Their proportion of the extracted fraction was 4.04 % (syringaldehyde) and 7.12 % (syringaresinol) and based on the non-extracted SPS 0.67 % and 1.19 %.

For the extract of **oak and chestnut wood** most of the detected peaks were related to sugars like for beech. However, the peak with the biggest proportion was detected to be ellagic acid with a proportion of 7.3 % for chestnut and 8.4 % for oak based on the extracted fraction, which corresponded to 1.1 % for both based on the non-extracted material. Next to ellagic acid, the gallic acid was detected, but in lower proportion. With 1.9 % gallic acid in the extract of chestnut wood the proportion was double the amount detected for oak with 0.9 %. Recalculated to the non-extracted material this discrepancy was consistent and thus in line with literature showing higher proportions of gallic acid in chestnut than in oak (Sanz et al., 2010). Further, a lignan was detected in the extract of oak and chestnut wood, which could be identified as lyoniresinol with proportions of 1.2 % (oak) and 1.0 %(chestnut), which is equal to 0.16 % and 0.15 % based on non-extracted material.

Focusing more on the extract of the **SPS** of oak and chestnut some discrepancies raised. The different share of gallic acid detected between oak and chestnut wood was not detected for the SPS, where gallic acid had a proportion of 0.7 % and 0.6 % for chestnut and oak extract. The ellagic acid was also detected in the SPS with 2.1 % for oak and 2.7 % for chestnut based on the extract. Based on the non-extracted SPS the ellagic acid accounted to 0.5 % and 0.6 % for oak and chestnut and thus the proportions of ellagic acid were lower in the SPS than the wood.

The lignan lyoniresinol was not detected in the extract of the SPS. Therefore, this substance seemed to be present as such in the wood and was no reaction product of the SE process. This substance was also detected in the LPS of oak, which indicated that the lignan became dissolved during the SE process. In contrast to the lyoniresinol, the lignan syringaresinol was detected in the SPS extract of oak and chestnut with proportions of 1.2 % and 1.1 % of the extracted fraction. Additionally, 0.6 % of syringaldehyde were detected in the SPS extract, which was not the case for the wood extract of oak and chestnut.

Analyses by pyrolysis GC/MS

The analyses of **all samples** showed different phenol or lignin related fragments as major proportion. The substance with the biggest share within the wood and the SPS extracts for beech and oak/chestnut was clearly syringol. There were further substances, which seemed to be present simultaneously in the Ace/H_2O extract of wood and the SPS for all species. For instance 1,2-benzendiol, 3-methoxy or guaiacyl - $CH=CH_2$ as well as syringyl - CHO (syringaldehyde), next to others.

From the presence of substances there were no big **differences** detected for oak and chestnut, but compared to beech some differences were obvious, especially for the **SPS**. The substances, detected for oak and chestnut (SPS), but not for the SPS of beech were mainly related to guaiacyl - residues with different chain lengths and functional groups. For example, guaiacyl - CH_2CH_3, guaiacyl-CHO (vanillin) or guaiacyl - CH_2-CH_2-CH_3 were detected as well as two ketons guaiacyl - CO-CH_3 and guaiacyl - CH_2-CO-CH_3. However, these substances were partly observed in beech wood. Solely one syringyl- derivative (syringyl - CH_2-CH_2-CH_3) was detected in oak and chestnut, but not for beech SPS. Similar effects were detected for a not clearly identified stilbene and two steroids (stigmasta-3,5-diene and sitosterol), which were detected in beech wood, but not in the SPS. The stilbene and the steroid stigmasta-3,5-diene were also present in oak and chestnut wood, but solely the stilbene could be detected in the SPS. Further, the substance syringyl - CH_2-$CH=CH_2$ could be detected in beech wood and SPS, but were absent in oak and chestnut SPS

Further analyses with **on-line derivatization** and analysis by pyrolysis GC/MS showed some syringyl- and guaiacyl- derivatives similar to the already detected ones. However, it also showed gallic and ellagic acid in the extract of wood and the SPS of oak and chestnut as well as the lignan lyoniresinol in the wood extract. Interestingly, the acids and the lignan were also detected during the GC/MS measurements without pyrolysis, but not during pyrolysis GC/MS measurements without silylation. Table 25 presents the identified substances of the different species detected by the different (pyrolysis) GC/MS analyses.

Considering the limitations of the methods, it is very likely that just a proportion of the substances in the extracts could be detected by the applied methods. However, these analyses showed that traces of tannins like gallic acid and ellagic acid were present in wood and the SPS of oak and chestnut db, but not in beech db. Additionally, the extracted fraction of the SPS contained a high variety of syringyl- and guaiacyl- derivatives, which indicated the presence of lignin degradation products. Due to the less condensed structure of S-lignin (Mai & Zhang, 2023) it is more easily cleaved during SE process, which explains the high amount of detected syringyl-fragments. Further, in the SPS extract of oak and chestnut some proportions of guaiacyl- derivatives were present, which were not observed for beech. This potentially indicated a different lignin structure in the SPS of oak and chestnut compared to beech.

Regarding a comparison with the FTIR analyses, the spectra of oak and chestnut wood (Figure 42) showed a wide peak around 1330 cm^{-1}, which can be attributed to syringyl units (Fengel & Wegener, 2003; Schwanninger et al., 2004), but is also described as characteristic for

hydrolysable tannins (Falcão & Araújo, 2013). This signal became sharper in the spectra of the SPS extracts (Figure 46) and also for beech the signal was sharper. These findings agree with the high proportions of S-lignin fragments detected during the pyrolysis GC/MS for all species. Additionally, the signal around 1270 cm^{-1}, which was assigned to G-lignin (Fengel & Wegener, 2003; Schwanninger et al., 2004) seemed to be more intense for oak and chestnut than for beech in the spectra of the SPS extracts. This agreed with the higher share of G-lignin fragments detected during the pyrolysis GC/MS measurements of oak and chestnut. Additionally, the detected decrease of ellagic acid in the SPS extract of oak and chestnut during the GC/MS measurements, supported the assumption that the proportions of hydrolysable tannins, as detected in wood, changed due to the SE-process.

Overall, the characterization by (pyrolysis) GC/MS measurements revealed the presence of tannin fragments as well as sugars and various lignin fragments and agreed with the characterization of the FTIR spectra. The detected presence of tannins and the increased proportion of G-lignin fragments in oak and chestnut SPS extracts indicated that the Ace/H_2O extraction removes a fraction, which potentially adsorbs or inhibits enzymes. A higher affinity of G-lignin for the adsorption of enzymes was reported by Guo et al. (2014) and Tejirian and Xu (2011) as well as Ximenes et al. (2011) observed strong inhibitory effects for tannic acid as a model compound for tannins. These results thus contributed to research question 3.2, but needed supportive analyses to show the actual effect.

Table 25: Substances found in the Ace/H_2O extract of wood and SPS of beech, oak and chestnut debarked applying different GC/MS methods

Ace/H_2O extract						Substances	Method	
Wood			**SPS**					
Beech (db)	Oak (db)	Chest (db)	Beech (db)	Oak (db)	Chest (db)			
						Phenol derivatives		
✓	---	---	---	---	---	Phenol	Pyr	
✓	✓	✓	✓	✓	✓	3-Methoxy-1,2-benzenediol	Pyr	
✓	---	---	---	---	---	Phenol derivative	Pyr	
✓	✓	✓	✓	✓	✓	Phenol derivative	Pyr	
✓	✓	---	✓	---	---	3,4,5-trimethoxy phenol	Pyr	
✓	✓	✓	✓	✓	✓	2-Methoxy-5-methylphenyl	Pyr	
						Guaiacyl-derivatives		
✓	✓	✓	✓	✓	✓	Guaiacol	Pyr	Pyr + silyl
✓	✓	✓	---	✓	✓	Guaiacyl -CH_2-CH_3	Pyr	
✓	✓	✓	---	✓	✓	Guaiacyl -CH_2-CH_2-CH_3	Pyr	
✓	✓	✓	✓	✓	✓	Guaiacyl -CH=CH_2	Pyr	
✓	---	---	✓	✓	✓	Guaiacyl -CH=CH-CH_3	Pyr	
---	✓	✓	---	✓	✓	Guaiacyl -CHO (Vanillin)	Pyr	Pyr + silyl
✓	✓	✓	---	✓	✓	Guaiacyl -CO-CH_3	Pyr	
✓	✓	✓	---	✓	✓	Guaiacyl -CH2-CO-CH_3	Pyr	
✓	---	---	---	---	---	Guaiacyl -CH_2-CH_2-CH_2OH	Pyr	
✓	---	---	---	---	---	Guaiacyl -CH=CH-CH_2OH (Coniferyl alcohol)	Pyr	Pyr + silyl

Continued ↓

						Syringyl- derivatives			
✓	✓	✓	✓	✓	✓	Syringol	Pyr	Pyr + silyl	
✓	---	---	---	---	---	Syringyl -OH (Hydroquinone)	Pyr		
✓	✓	✓	✓	✓	✓	Syringyl -CH_2-CH_3	Pyr		
---	---	---	---	✓	✓	Syringyl -CH_2-CH_2-CH_3	Pyr		
✓	✓	✓	✓	✓	✓	Syringyl -CH=CH_2	Pyr		
✓	✓	✓	✓	✓	✓	Syringyl -CH=CH-CH_3	Pyr		
---	---	---	✓	---	---	Syringyl -CH=CH-CH_3	Pyr		
✓	---	---	✓	---	---	Syringyl -CH_2-CH=CH_2	Pyr		
✓	✓	✓	✓	✓	✓	Syringyl -CHO (Syringaldehyde)	Pyr	Pyr + silyl	GC/MS + silyl
---	---	---	✓	---	---	Syringyl -CH_2-CHO	Pyr		
✓	✓	✓	✓	✓	✓	Syringyl -CH=CH-CHO	Pyr	Pyr + silyl	
---	✓	✓	✓	✓	✓	Syringyl -CO-CH_3	Pyr		
✓	✓	✓	✓	✓	✓	Syringyl -CH_2-CO-CH_3	Pyr		
✓	---	---	✓	---	---	Syringyl -CH=CH-CH_2OH	Pyr	Pyr + silyl	
						Others			
✓	---	---	---	---	---	Organic acid (n.i)		Pyr + silyl	GC/MS + silyl
✓						Palmitic acid		Pyr + silyl	
---	✓	✓	---	✓	✓	Gallic acid		Pyr + silyl	GC/MS + silyl
---	✓	✓	---	✓	✓	Ellagic acid		Pyr + silyl	GC/MS + silyl
---	---	---	✓	✓	✓	Syringaresinol (Lignan)		Pyr + silyl	GC/MS + silyl
✓	---	---	---	---	---	Catechin (Flavonoid)		Pyr + silyl	GC/MS + silyl
---	✓	✓	---	---	---	Lyoniresinol (Lignan)		Pyr + silyl	GC/MS + silyl
✓	✓	✓	---	✓	✓	Stilbene (n.i.)	Pyr		
✓	✓	✓	---	---	---	Stigmasta-3,5-diene (Steroid)	Pyr	Pyr + silyl	
✓	---	---	---	---	---	Sitosterol (Steroid)	Pyr	Pyr + silyl	

✓ = substance detected

✓ = marks differences between the species

✓ = marks peaks with high peak proportion

--- = substance not detected

n.i. = not clearly identified

Pyr = pyrolysis GC/MS

Pyr + silyl = pyrolysis GC/MS with on-line silylation

GC/MS = liquid mode with silylation

5.20 Effect of Ace / H_2O extraction

This chapter focuses on the effect of Ace/H_2O extraction in relation to enzymatic hydrolysis and the adsorption or inactivation of enzymes. Further, the combination of the extraction effect and the Tween20 effect was investigated. The analyses thus included different enzymatic hydrolysis scenarios and the screening of the protein composition in the supernatant by SDS-PAGE.

5.20.1 Effect of Ace / H_2O extraction and Tween20 on enzymatic hydrolysis

For the enzymatic hydrolyses SPS material (wet) from debarked beech and oak was extracted directly with Ace / H_2O (described in chapter 4.2.9). Material from chestnut was not included, as the previous analyses showed a similar chemical composition to oak. Enzymatic hydrolyses were performed comparative for non-extracted and extracted material. The enzymatic hydrolysis ran for 48 h following EH scenarios 9 & 10 (chapter 4.2.9).

For beech, the glucan conversion of **non-extracted and extracted material** was very similar with 48.5 % and 46.9 % (Figure 48), suggesting that the extraction has no extra impact on the conversion of SPS from beech. In contrast, for the conversion of SPS from oak, the extraction showed a strong effect. While the glucan conversion was 14.7 % for non-extracted material, it was 28.3 % for the extracted material, which corresponds to an increase of 92.5 %.

This strong improvement led to the assumption that the chosen extraction method successfully removed or at least decreased compounds from the SPS, hampering the enzymatic digestibility. However, it has to be considered that the SPS was washed after the extraction to remove the acetone from the material. Therefore, the observed positive effect of the extraction was likely due to a combination of the washing effect (+18.5 %) (chapter 5.17) and an extraction effect. Despite the strong positive effect of the applied extraction, the performance gap between beech and oak remained.

The combination of the washing effect and the Tween20 effect did not show additional improvements (refer to chapter 5.17 and Figure 37), but the combination of the extract effect and the Tween20 effect still needed to be tested. Therefore, the enzymatic hydrolyses were repeated comparing **non-extracted and extracted material** with the **addition of Tween20** (EH scenarios 11 and 12). This time the extraction showed a positive effect for beech by increasing the glucan conversion rate from 76.4 % to 84.8 %, but there was a notable standard deviation for the non-extracted samples, which needed to be considered (Figure 48).

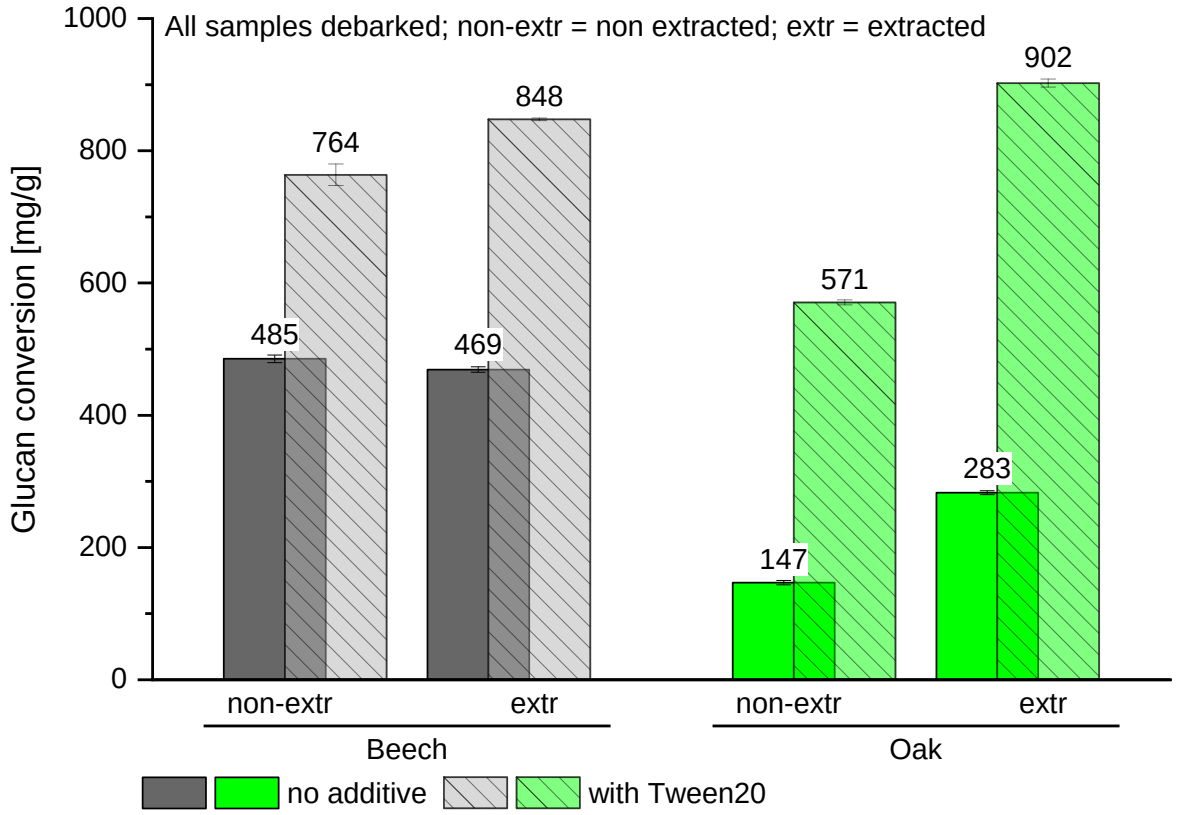


Figure 48: Effect of Ace / H_2O extraction and the additive Tween20 on the glucan conversion [mg/g] after 48 h of enzymatic hydrolysis.

For oak, the positive effect of Tween20 for the non-extracted material was clearly visible, similar to the experiments in chapter 5.15. However, the improvement for the extracted material was outstanding. The combination of the Ace/H_2O extraction effect and the Tween20 effect increased the glucan conversion rate of oak db from 57.1 % (non-extracted, Tween20) to 90.2 % (extracted, Tween20), which corresponded to an improvement of 58.0 %. With a conversion rate of 90.2 % oak even surpassed beech within this experimental setup. Compared to the conversion rate of the non-extracted material without Tween20 the conversion rate was improved by 513.6 % and compared to the extracted material without Tween20 it was improved by 219.9 %. This outstanding improvement clearly showed that the extraction with Ace/H_2O removed the inhibitory fraction of the SPS from oak almost quantitatively. In combination with Tween20 the non-productive binding was prevented as well, resulting in a strongly increased glucan conversion rate.

Comparing the results with the first experiments using Tween20 (chapter 5.15) the current results of the experiments show a slightly lower conversion in the controls and a slightly higher conversion if Tween20 is added. This discrepancy between the experiments is probably based on the different experimental designs regarding work volume, flasks, kind of mixing and sample taking. However, the value range of the experiments is totally in line with each other.

Therefore, the influencing factor of non-productive adsorption had the strongest impact for oak and chestnut, but the removal of the Ace/H_2O soluble fraction was an additional key factor for a profitable conversion. These results thus contribute to research questions 3.2 and 3.3.

However, at this rate it could not be clarified, if these effects interact synergistically or complementary. On the one hand, the removal of the Ace/H_2O soluble fraction potentially changed the substrate surface to an extent, which enhanced the effectiveness of Tween20,

for instance by improved accessibility of the surface for the Tween20 molecules. On the other side, the extraction could remove compounds, which inactivate enzymes and thus act complementary to Tween20.

5.20.2 Analysis of the free protein after enzymatic hydrolysis

To investigate the effect of the Ace/H_2O extraction and of Tween20 in more detail, the free protein in the supernatant was analyzed by SDS-PAGE and staining with Coomassie® Blue. For these analyses the supernatant after 2 h of enzymatic hydrolysis, following the conditions of EH scenario 9 - 12, was concentrated 10 - fold to reach a theoretical protein concentration of 1.0 mg/ml. The reaction was disrupted after 2 h to ensure that the amount and composition of enzymes for oak and beech samples displayed by SDS-PAGE represented the same state of reaction. For instance, after 48 h the glucan conversion would be nearly finished for oak (extracted, Tween20), but not for beech, which would result in more free enzymes in the supernatant of oak, as there is no cellulose left for the cellulases to bind to. Additionally, prior experiments with non-concentrated supernatants and silver staining showed detectable protein amounts, but also that the staining with $AgNO_3$ was influenced by soluble substances within the SPS of oak. This influence led to a general darker staining background and smearing. Therefore, a concentration step and the staining with Coomassie® Blue were chosen.

The here used enzyme blend (Cellic®CTec2) by Novozymes® was described to contain a blend of cellulases, ß-glucosidases and hemicellulases (Novozymes, 2012). The main enzymes produced by *T. reesei* as a well studied cellulolytic microorganism are cellobiohydrolases (CBH) with 55 kDa (CD+CBM), the endoglucanase Cel7B with a size of 55 kDa, the ß-glucosidase Cel3A with 81 kDa as well as the xylanase XynIV with 55 kDa and a mannanase with 53 kDa. Additional xylanases, like X1 with 110 kDa, X2 with 100 kDa or X3 with 80 kDa could be present (Lee et al., 2022). Further, endoglucanases with a size of 100 kDa (E1) or 87 kDa (E2, Cel74A) next to smaller endoglucanases down to 10 kDa were reported by Lee et al. (2022). Their investigation of the enzyme adsorption on different lignin types showed a higher adsorption for proteins with high molecular weights > 70 kDa. For example an adsorption rate of 52 % at oak lignin for ß-glucosidase Cel3A (81 kDa) and 15 % for cellobiohydrolases (55 kDa) was found. Interestingly, it was also found by the same researchers that the adsorption affinity of the cellobiohydrolases correlated positively with the S/G lignin ratio, detected for different lignin sources (Lee et al., 2022). Ko et al. (2015b) reported a loss of the protein bands for ß-glucosidases during SDS-PAGE of free protein after incubation with isolated lignins, which correlated to the detected strong decrease of ß - glucosidase activity. Further, Rahikainen et al. (2011) found and decreased activity for endoglucanase (60 %) and ß-glucosidase (> 80 %) for adsorption experiments with lignin residues from enzymatic hydrolysis resulting in a total protein adsorption of 95 %.

Figure 49 presents the SDS-PAGE run comparing the non-extracted with the extracted scenarios and the Tween20 scenarios for beech and oak, next to a protein marker and an enzyme blank. Transferring the reported enzyme sizes from Lee et al. (2022) to the here presented pattern of protein sizes the first band > 100 kDa could potentially belong to an endoglucanase (EG) or xylanase. Likely the bands between 100 and 63 kDa were assigned to ß-glucosidases (BGL) and below 63 kDa to CBHs and EGs as well as low molecular xylanases.

Focusing first on the **non-extracted** and **extracted** scenarios for **beech**, the protein band pattern did not vary strongly between the two scenarios. However, the protein bands between 130 – 63 kDa appeared slightly less intense for the extracted material. Additionally, the slight band below 28 kDa in the non-extracted beech sample, was not visible in the extracted sample.

These small differences would correlate with the observed slightly lower glucan conversion rate for beech extracted. Overall, comparing the protein band pattern of the samples with the enzyme blank, which has a similar initial protein concentration, showed that also for beech some enzymes were removed from the supernatant, due to the lower band intensity.

For the samples from **oak**, similar to beech the non-extracted scenario did not differ strongly from the extracted scenario. However, compared to beech notable differences were observed. The bands at the top between 130 – 75 kDa were completely missing in the non-extracted and the extracted scenario of oak. Additionally, there were no bands detected between the band below 63 kDa and the wide band above 35 kDa, which should be present in relation to the enzyme blank. The bands below 28 kDa were also completely missing in both scenarios. Solely slightly more intense bands around 63 and 35 kDa differed the extracted from the non-extracted oak sample, which could be assigned to EGs and CBHs or xylanases (Lee et al., 2022). The strongly decreased band intensities compared to the enzyme blank and beech correlated with the observed low glucan conversion rates for oak. This implicates a strong reduction of free enzymes in solution and thus agreed with the previous results. Additionally, the extraction seemed not to influence this reduction of free proteins itself.

Switching to the scenarios including **Tween20**, for **beech** clearly the band intensity increased notably, especially for the two bands above 63 kDa. Additionally, a complete new band appeared directly above the wide band around 35 kDa, which was not present in the scenarios without Tween20. One of the two protein bands below 28 kDa present in the enzyme blank was now also visible in the beech samples. However, comparing the non-extracted and extracted scenario with Tween20 the protein band patterns were similar, as it was the case for the scenarios without Tween20. This strong increase in band intensity indicated that the amount of free protein increased for both scenarios. Solely, the intensity of the band above 63 kDa and at 28 kDa appeared slightly more intense in the extracted scenario, which correlated with the slightly higher glucan conversion rates for extracted beech with Tween20.

For **oak** with **Tween20** the protein band patterns between the two scenarios were more differentiated. The non-extracted sample with Tween20 showed an increased band intensity and some more protein bands between 63 kDa and 28 kDa compared to the scenarios without Tween20. This indicated an increased proportion of free enzymes, which could be CBHs, EGs and xylanases (Lee et al., 2022) However, the proteins >75 kDa were not present, similar to the scenarios without Tween20. These protein bands solely appeared in the scenario with extracted oak material in combination with Tween20. These protein bands are probably attributed to endoglucanases and ß-glucosidases. The reported high molecular xylanases from *T. reesei* (Lee et al., 2022) were more unlikely, as the main effect was seen for the glucan conversion. One theory to explain these findings could be that in non-extracted oak material the enzymes above and around 75 kDa were inactivated. With the extraction step, the compounds, which inactivated these enzymes were removed and thus these enzymes were no longer inactivated. However, they were non-productively adsorbed, which was further prohibited by the addition of Tween20. Therefore, the two effects seem not to work synergistically but complementary. Further, the effect was species-specific, as for beech the protein band pattern did not change, but the intensity mainly due to the presence of Tween20. For beech the non-productive adsorption thus seemed to play the major role, whereas for oak the Ace/H_2O extraction is a crucial additional factor next to the non-productive adsorption of enzymes. Overall, these results contributed to research questions 3.2 and in combination with the results from the chapters 5.19 and 5.20 these findings contribute to research question 3.1 and strongly to 3.3.

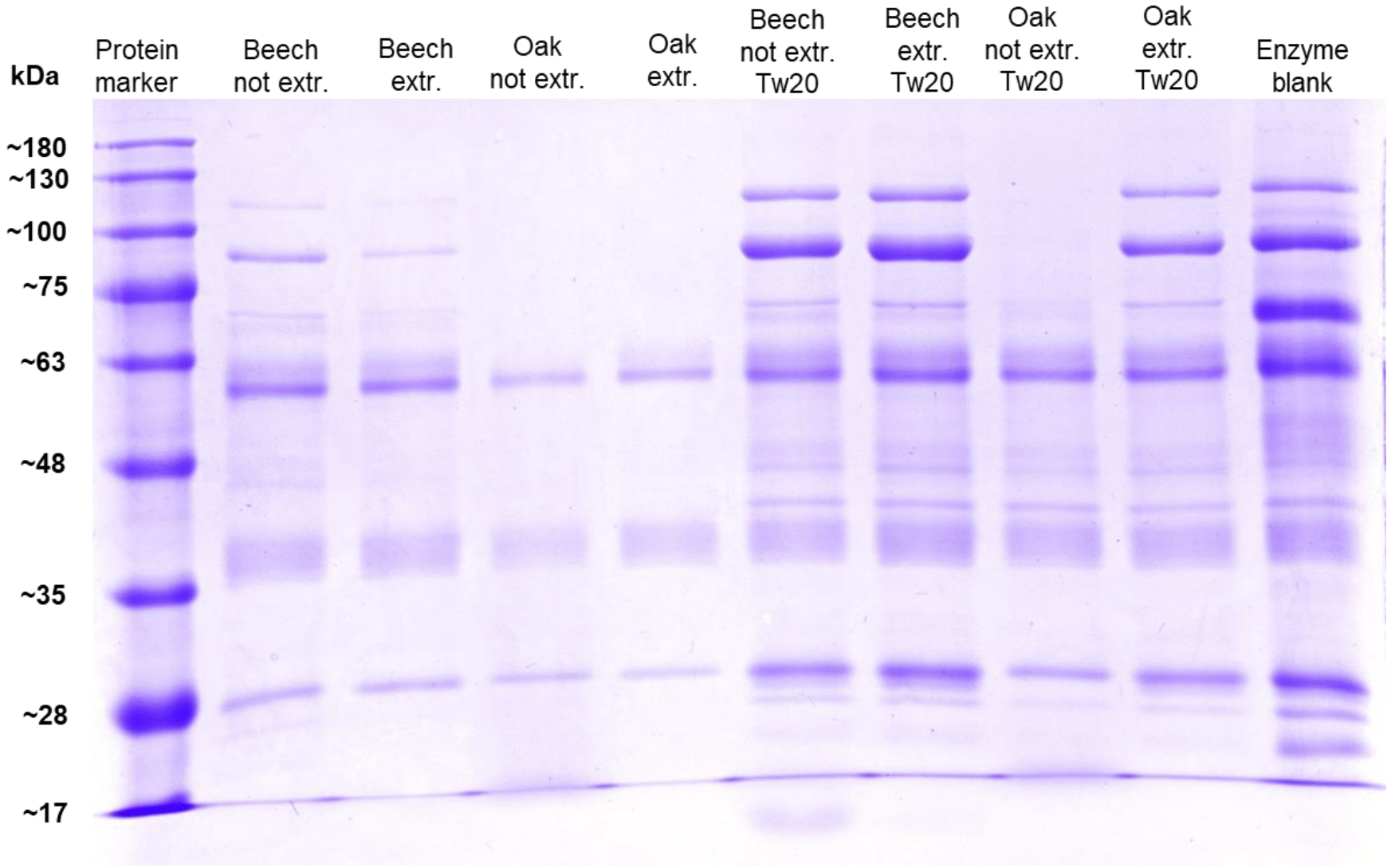


Figure 49: Size related separation of the proteins in the supernatant after 2 h of enzymatic hydrolysis by SDS-PAGE and Coomassie® Blue staining.

6. General discussion and Outlook

This thesis presents an experimentally funded analysis of material flows and the suitability and potentials of different hardwoods for biorefinery processes. Species-specific influencing factors on the enzymatic hydrolysis were investigated and strategies to improve the glucan conversion were studied. Four different hardwood species were included in the research and two scenarios “debarked (db)” and “with bark (wb)” were compared. Additionally, a mixed sample (db) with 60 % beech, 20 % ash and 20 % oak was analyzed. Within a primary refining step the wood chips were pretreated by steam explosion under acidic conditions (SE), resulting in a liquid and a solid process stream (LPS; SPS). The SPS was further converted by enzymatic hydrolysis (EH) to produce monomeric glucose as main platform.

Part 1: Species usability, component distribution and influence of bark

The characterization of the raw materials, showed differences between the tree species regarding the content and composition of sugars, extractives, lignin and minerals. The SE process removed mainly the hemicelluloses and the sugar content in the SPS increased relatively from solid wood to the SPS by 28 – 42 %, calculated on non-extracted basis. However, the suitability of the tree species for the production of glucose by EH was independent on the glucan content, but was strongly dependent on the tree species. Especially, between beech/ash and oak/chestnut the performance gap was considerable. Remarkably, already the small proportion of 20 % oak within the mixed sample decreased the performance notably, which proved a sharp hampering effect within the oak material. This separation within the tree species correlated with the content of hot water extracts, which were low for beech/ash and high for oak/chestnut. Additionally, it correlated with the respective FTIR analyses of these extracts, which showed a similar signal pattern for beech/ash and oak/chestnut. These differences were mainly due to the presence of tannin-related substances in the extracts of oak and chestnut. The presence of bark decreased the glucan content in negative correlation to the bark proportion. Simultaneously, the proportion of acid insoluble residues, as well as the extract content and the proportion of minerals increased. In case of enzymatic hydrolysis bark reduced the conversion ≥ 10 %, except for beech, where bark had no negative effect.

There are two main aspects of suitability. The first aspect is the proportion of usable products per raw material unit. In the here presented case the glucan was the component of interest. However, for example the extractives as the proportion with the highest variability between the tree species influenced the glucan content. Further, bark also affected the chemical composition and it would be necessary to evaluate the changes within the component share against the economic benefits of savings from eliminating debarking processes including machinery, storage and downstream processing to rate the profitability. The second aspect is the real usability of the target component. For instance in the chase of oak and chestnut the glucan content was not usable due to inhibitory effects of the species-specific wood extractives during EH. Therefore, to evaluate the conversion efficiency of hardwood species in biorefineries, the focus should be on extractives chemistry and composition.

The comparison of the water-soluble fraction from wood with the chemical composition of the LPS showed that the species-specific “fingerprint” regarding wood extractives partly remained after the SE process. However, additional substances were present in the LPS like lignin and sugar degradation products as well as different extractives, which were not detected in the hot water extracts from wood. The presence of bark did not change the composition of species-specific substances within the LPS, except for ash. Nevertheless, the influence of bark was detectable, by quantitative changes of the individual components for all species. Further, the species-specific composition of hemicellulosic sugars stayed detectable over the process as

another “fingerprint”. The LPS thus contained the main proportion of species-specific wood components. However, as shown during the EH also the SPS seemed to be still influenced by the tree species. In the SPS next to the glucan content also the proportion of acid insoluble substances increased relatively during the SE dependent to the tree species.

These results lead to some suggestions. First, these composition analyses make the distribution of species-specific extractives or other compounds partly predictable and potentially transferable to other tree species. Additionally, these can be already examined by analyzing the raw material at least to some extent. Second, the detected components should be seen as additional value added products available for utilization. If these would be used in upstream processes, potential disadvantage by a lower proportion of the main target product could be balanced. Further, the production of rare but valuable components like complex organic substances for example with a specific stereochemistry could also be advantageous for the positioning on the market. For instance, the detected hemicellulosic sugars and their derivatives are demanded substances for the pharmaceutical, the cosmetic or the food industry and can, for example, be upgraded as alternative sweeteners (Mäki-Arvela et al., 2011). Sugar degradation products, although not the main target products within this thesis could create potentially high additional value as well. Acetic acid is an important ingredient for the food industry. For furfural, as a degradation product of C_5 sugars, about 80 different derivatives with values for different industries are known. Additionally, HMF as a sugar degradation product of C_6 sugars is a platform chemical to produce polymers or biofuels. A further degradation of HMF results in levulinic acid a promising platform chemical for polymer materials or animal feed next to others (Gómez Millán et al., 2019). Regarding the extractives and lignin degradation products for instance, vanillin could be used as flavoring essence and has potential to substitute fossil based aromatic compounds in the production of polymers (Fache et al., 2016). Further, the lignan syringaresinol is known for its inflammatory and antioxidant properties and there is also some research on using syringaresinol as renewable substitution for the fossil based bisphenol A as important building block for epoxy-amine resins (Janvier et al., 2017; Li et al., 2020). The lignan lyoniresinol detected in oak, shows some antimicrobial or anti-oxidative effects (Lee et al., 2005). Next to medicinal effects, lignans also play a role in the seasoning of wine. Cretin et al. (2015) for example showed that the different stereoisomers of lyoniresinol extracted out of oak wood resulted in different tastes, from bitter to neutral to sweet. Further, the substance group of hydrolysable tannins has great potential for the pharmaceutical industry due to their antimicrobial, anti-inflammatory and antioxidant properties. For example, ellagitannins seem to inhibit some transcript sites and the adsorption of viruses on the cell surface, thus inhibiting the spread of the HIV virus (Okuda et al., 1989). Lastly, also for the substances detected solely in ash with bark, the fraxinol derivatives have a utilization potential as optical brighteners (Drosky et al., 2014). The third suggestion is that the here applied analyses likely did not uncover the whole potential of the species due to methodical limitations. Therefore, a deeper investigation of species-specific substances focused on one tree species or one specific substance class would enable to choose more suited analytical methods and could reveal more of the further utilization potentials.

As an additional aspect, this thesis gave an overview on accumulation and leaching of minerals as reference for a holistic plant management and a potential utilization of the mineral fraction. Further, the specific challenges to include bark into the raw material portfolio were worked out, which mainly increased the amounts of the individual minerals. Potassium, sodium, manganese or phosphor were completely leached into the LPS. Therefore, this process stream can be the preferable source to utilize rare minerals like phosphor and manganese, which are important for plant fertilizing. Further, the detected leaching pattern pointed out that the acidic conditions of the process were preferable for the further conversion of the SPS to glucose by enzymatic hydrolysis as the removal of minerals under acidic conditions was described as beneficial (Aston et al., 2016; He et al., 2014; Hörhammer et al., 2018). However, some

detected elements like the proportions of Fe in the SPS, especially for the wb samples potentially could be seen critical, too. Liu et al. (2010) detected a negative influence of Fe^{3+} during enzymatic hydrolysis, but this would depend on the ionic form of the Fe in SPS, which was not determined in this thesis.

Part 2: Impact of factors influencing enzymatic hydrolysis and species-specific patterns

The examination of different influencing factors on enzymatic hydrolysis disclosed, which factors are most important to evaluate the performance between different tree species. The discussion of the results from the particle size distribution and accessibility measurements, however showed that the general interconnection of macroscopic and microscopic wood structure with the effectiveness of the pretreatment is not completely clear, especially with the integration of bark. The differences between ring-porosity and diffuse-porosity in terms of permeability or the influence of fiber proportions, especially in bark would need further research to understand the interconnections completely. However, the particle size distribution and the accessibility of the substrate for enzymes was shown to be less important to explain the differences between the tree species under similar process conditions. The positive effect of particle size reduction and disruption of the solid lignocellulosic tissues, which is emphasized as an important factor of pretreatment in literature (Lee et al., 2021; Wang et al., 2020), seemed to be fulfilled at this point. Additionally, the already low particle sizes probably decreased the importance of good pore accessibility due to the high surface area available for reaction and thus other influencing factors became dominant. The determination of the S/G lignin ratio showed a higher G-lignin content in oak and chestnut, which indicated a potential higher enzyme adsorption capacity for these species, which would agree with findings in literature (Guo et al., 2014; Lee et al., 2022). However, this approach needed supportive analyses to strengthen the assumption. Analyses of the effect of enzyme concentration revealed the importance to use low enzyme loads dependent on the glucan content of the material to prevent the masking of species-specific effects.

The non-productive binding of enzymes on the substrate was found to be an important factor to explain differences between the species and had the most informative value. This influencing factor affected all species db and wb. However, the effect intensity repeatedly grouped the tree species, as beech/ash showed a similar effect and oak/chestnut showed a much stronger effect. Additionally, a notably higher capacity to remove enzymes from the reaction medium was observed for oak and chestnut, which could be roughly halved, but not completely reversed by Tween20. Therefore, the addition of Tween20 could be one approach to equalize species-specific effects at least to a specific extent. The additive shortened the reaction time by roughly 24 h in relation to the conversion rate and reduced the necessary enzyme load by half, which is in line with findings in literature, for example in Eriksson et al. (2020). The non-productive adsorption was also influenced through the presence of bark by a decreased glucan conversion rate. The effect correlated with the bark proportion of the tree species, with little to no effect for beech and chestnut, but a strong effect for ash and oak. Therefore, wood-bark mixtures derived from trees with a thin bark seem to be beneficial, which leads to the suggestion that wood materials from thinnings or short rotation plantations would be sufficient as raw material for biorefineries. These assortments contain wood from young trees, which often have a thin and smooth bark due to the young development state of the bark. However, it has to be considered that these trees have a small average diameter and thus the ratio of wood to bark increases. An additional potentially beneficial aspect, is the smaller proportion of extractive rich heartwood within young trees.

Further, ellagic acid as specific substance in oak and chestnut did not show a hampering effect on EH. In contrast, an intensive washing of the SPS prior to EH to remove all water soluble substances showed a positive effect for oak and chestnut. However, the glucan conversion

rate did not reach the level of beech. The combination of the washing effect and the Tween20 effect also did not show any further improvement. Therefore, the water soluble compounds had no or a small impact on the enzymatic hydrolysis, but were likely not interconnected to species-specific effects.

Part 3: Species-specific effects and approaches to improve the enzymatic digestibility

Additional experiments with different Tween20 concentrations showed that the non-productive binding effect was limited to exhaustion. Considering the tested influencing factors, still the conversion rate of oak and chestnut could just be raised to ~ 55 % which was smaller than for beech. These aspects thus could not fully explain the low performance of oak and chestnut.

The hampering effect of oak and chestnut was focused on the water insoluble fraction of the SPS and was likely interconnected with their species-specific extractives. Therefore, this thesis used a novel approach to target the specific interactions of hydrolysable tannins during steam explosion (acidic conditions) and the following enzymatic hydrolysis. To do so, a tannin rich fraction was extracted with Ace/H_2O from the wood and the SPS of oak and chestnut as well as from beech as the reference material, which does not contain hydrolysable tannins. The analyses verified that the presence of hydrolysable tannins is the biggest difference between the tree species. Additionally, FTIR and (pyrolysis-) GC/MS measurements showed that the proportion of hydrolysable tannins decreased in the extracts of oak and chestnut due to the SE process. The composition of aromatic compounds changed as well and differs between beech and oak/chestnut. This could be possible reasoned in different polymerization and condensation reactions of lignin, sugar degradation products and tannins. Further, oak and chestnut extracts differed from beech by a higher presence of G-lignin fragments of different constitutions, which agreed with the detected differences in the S/G lignin ratio. However, the structural changes could not be clarified in detail and some more research for example by NMR measurements would be necessary to explain the structural changes. Further, the potential presence of tannins in the SPS after SE has to be considered during interpretation of literature data. For example, Lee et al. (2022) investigated the enzyme adsorption on lignin originated from oak next to others, which was produced by a “popping pretreatment”, comparable to the SE process, but the potential presence of tannin-related structures was not considered during interpretation.

The analysis of the effect of the Ace/H_2O extraction on the enzymatic hydrolysis showed no effect for beech, but a strong improvement (+92.5 %) of the glucan-conversion rate for oak from 14.7 % to 28.3 %. Thus, an Ace/H_2O extraction of hardwood species with obligatory heartwood can remove the specific fraction of the SPS, which inhibit the enzymatic hydrolysis. Remarkably, the combination of the extraction effect and the Tween20 effect improved the glucan conversion rate of oak further up to 90.2 %. The examination of the working mechanism between both effects by SDS-PAGE, revealed that the Ace/H_2O extraction removed the fraction from the SPS, which inactivated enzymes, especially >75 kDa and the addition of Tween20 prohibited the non-productive adsorption of these. The assumed crucial role of the species-specific characteristics were thus proved at least for oak.

It was shown for the first time that the extraction effect and the Tween20 effect work complementary. This process modification has the potential to increase the enzymatic digestibility of oak substrate to a profitable rate equal to or better than the reference material from beech. Therefore, to further develop this approach the effectiveness of the treatment needs to be tested for different tree species with comparable chemical characteristics. Additionally, different solvent combinations could be tested and the ratio of solid load and enzyme load needs to be adapted for an optional up-scaling.

7. Summary

The broad and comprehensive experimental design of this thesis, enabled an overview on the usability of different hardwood species and their bark for processes of the first refining step in lignocellulosic biorefineries. Species-specific effects and potentials as well as the respective influence of bark were analyzed to disclose the general distribution of the compounds within the process streams, evaluate glucan conversion rates and address the formation of additional valuable side products.

With a novel approach the behavior of hydrolysable tannins during the steam explosion under acidic conditions was investigated. This substance class and related structures were identified as species-specific for oak and chestnut and showed a strong hampering effect on enzymatic hydrolysis. Further, it was shown for the first time that a combination of an Ace/H_2O extraction prior to the enzymatic hydrolysis and an addition of the surfactant Tween20, neutralized this species-specific effect. These results have the potential to completely change the options to valorize lignocellulosic material of extractive rich hardwood species in combination with biological conversion processes in biorefineries.

Finally, Figure 50 presents an overview on the main outputs of this thesis structured by the respective research questions and process streams.

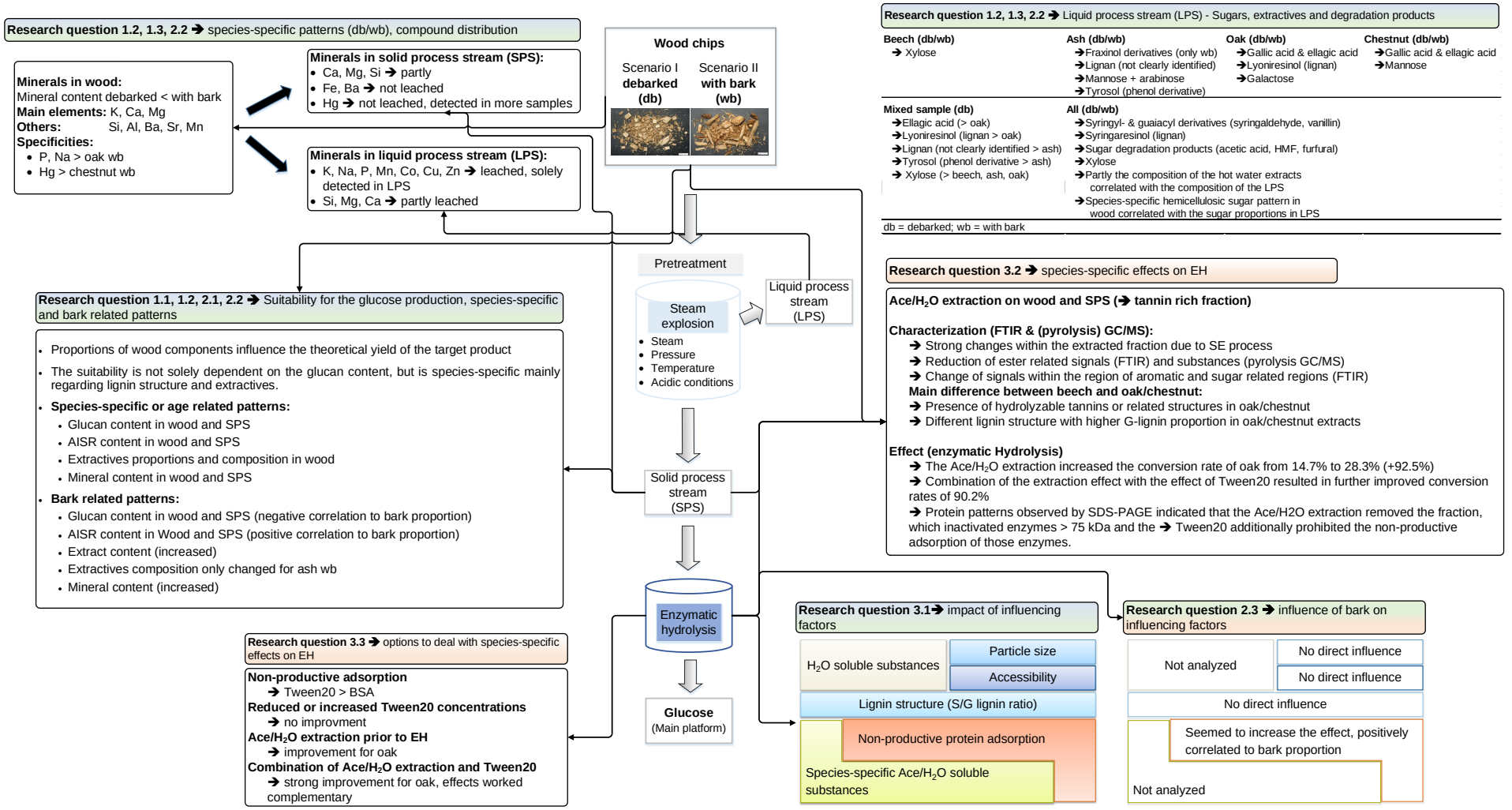


Figure 50: Main outputs of this thesis about utilization potentials for hardwoods as raw material for biorefinery processes

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Appendix

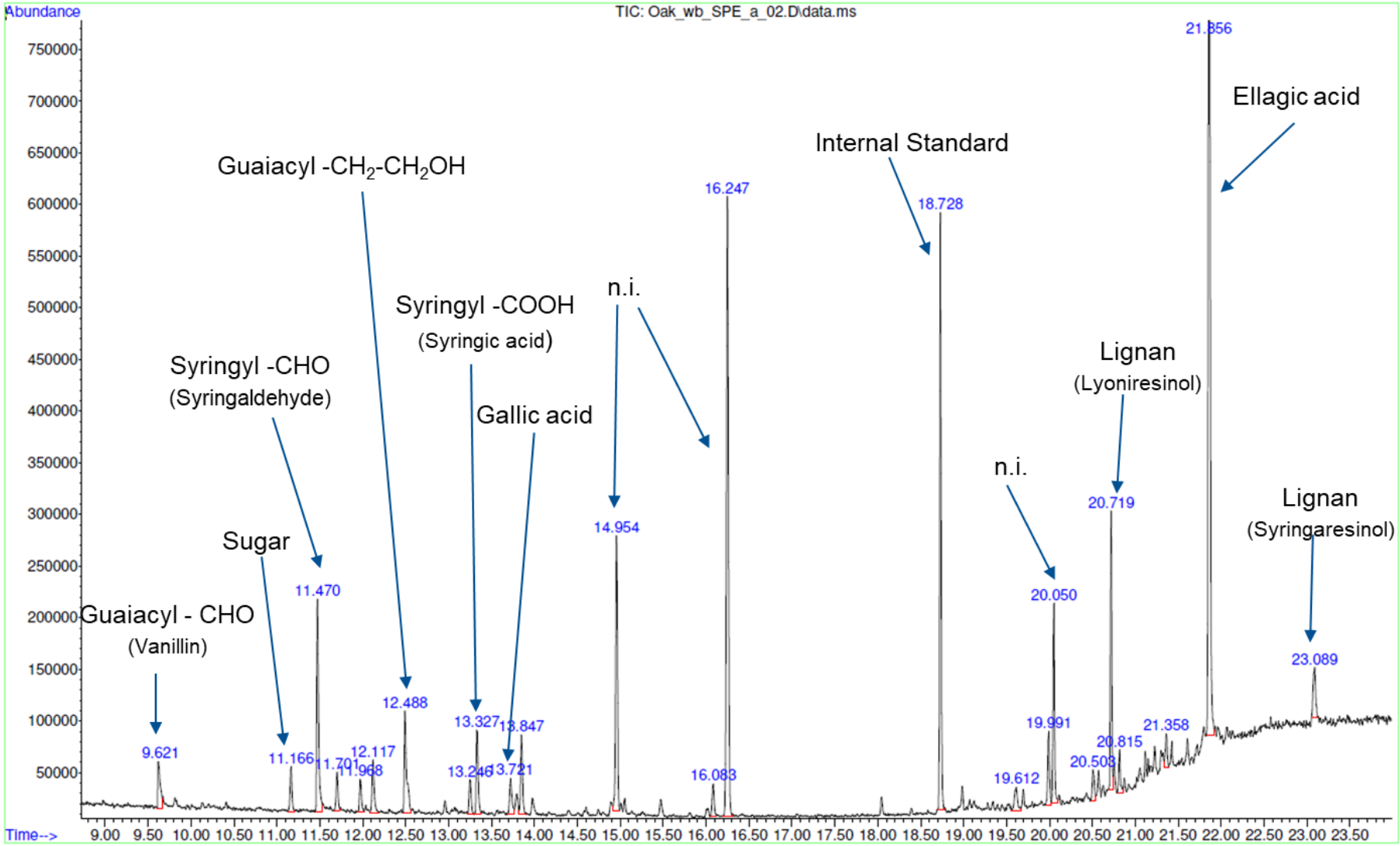

Figure 51: Representative chromatogram for the analysis of the eluate of the LPS after SPE at GC/MS, exemplary for oak wb (Chapter 5.8.2).

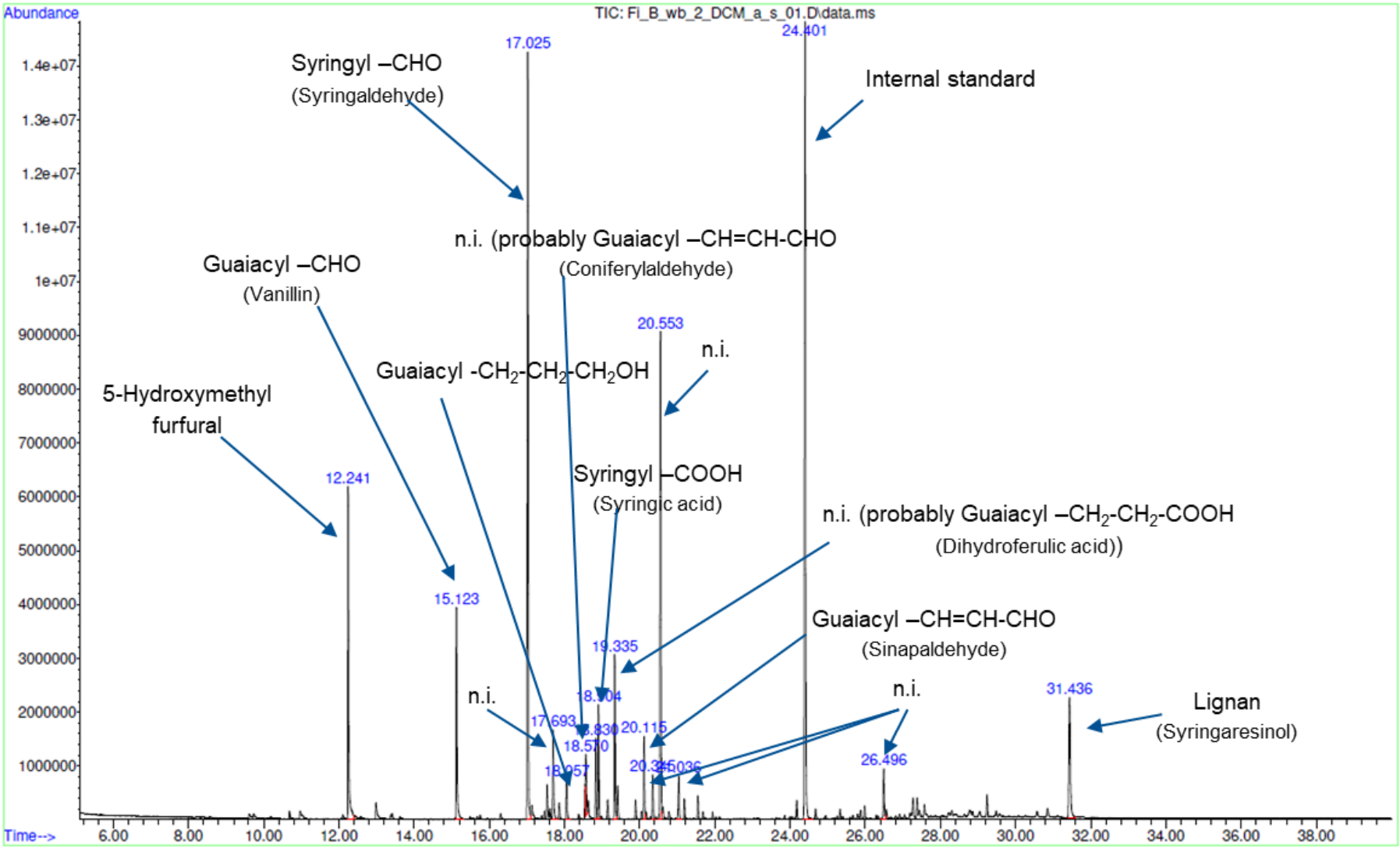

Figure 52: Representative GC/MS chromatogram of the LPS analyses at GC/MS after DCM extraction, exemplified for the LPS of beech wb (Chapter 5.8.2).

Table 26: Characteristic values and their standard deviation for the particle size distribution measurements (Chapter 5.11).

Sample		Smallest Particle Size [µm]	Biggest Particle Size [µm]	Mode [µm]	D10 [µm]	D90 [µm]	Slope of cumulative distribution *
Beech	db	0.30	180	45 (0.06; 1.60)	6.97 (1.23; 17.60)	83.06 (3.66; 4.41)	0.91
Ash	db	0.50	300	50 (0.12; 3.33)	7.39 (0.22; 2.94)	92.78 (8.04; 8.67)	0.87
Oak	db	0.30	180	40 (0.02; 0.59)	6.80 (0.08; 1.13)	76.25 (0.93; 1.22)	0.92
Chestnut	db	0.30	180	36 (0.05; 1.26)	6.10 (0.13; 2.16)	67.34 (3.28; 4.87)	0.91
Mix	db	0.30	600	63 (0.20; 6.01)	7.96 (0.41; 5.19)	130.30 (27.58; 21.17)	0.77
Beech	wb	0.04	600	56 (0.20; 5.83)	7.95 (0.56; 7.07)	124.66 (31.03; 24.89)	0.80
Ash	wb	0.04	600	71 (0.15; 4.87)	9.91 (0.58; 5.83)	194.10 (42.24; 21.76)	0.70
Oak	wb	0.04	242	53 (0.07; 1.84)	6.97 (0.14; 2.07)	97.24 (4.88; 5.02)	0.86
Chestnut	wb	0.30	180	45 (0.03; 0.70)	7.01 (0.10; 1.36)	80.27 (1.84; 2.29)	0.91

(SD; relative SD) * calculated between 10 and 100 µm db =debarked wb = with bark

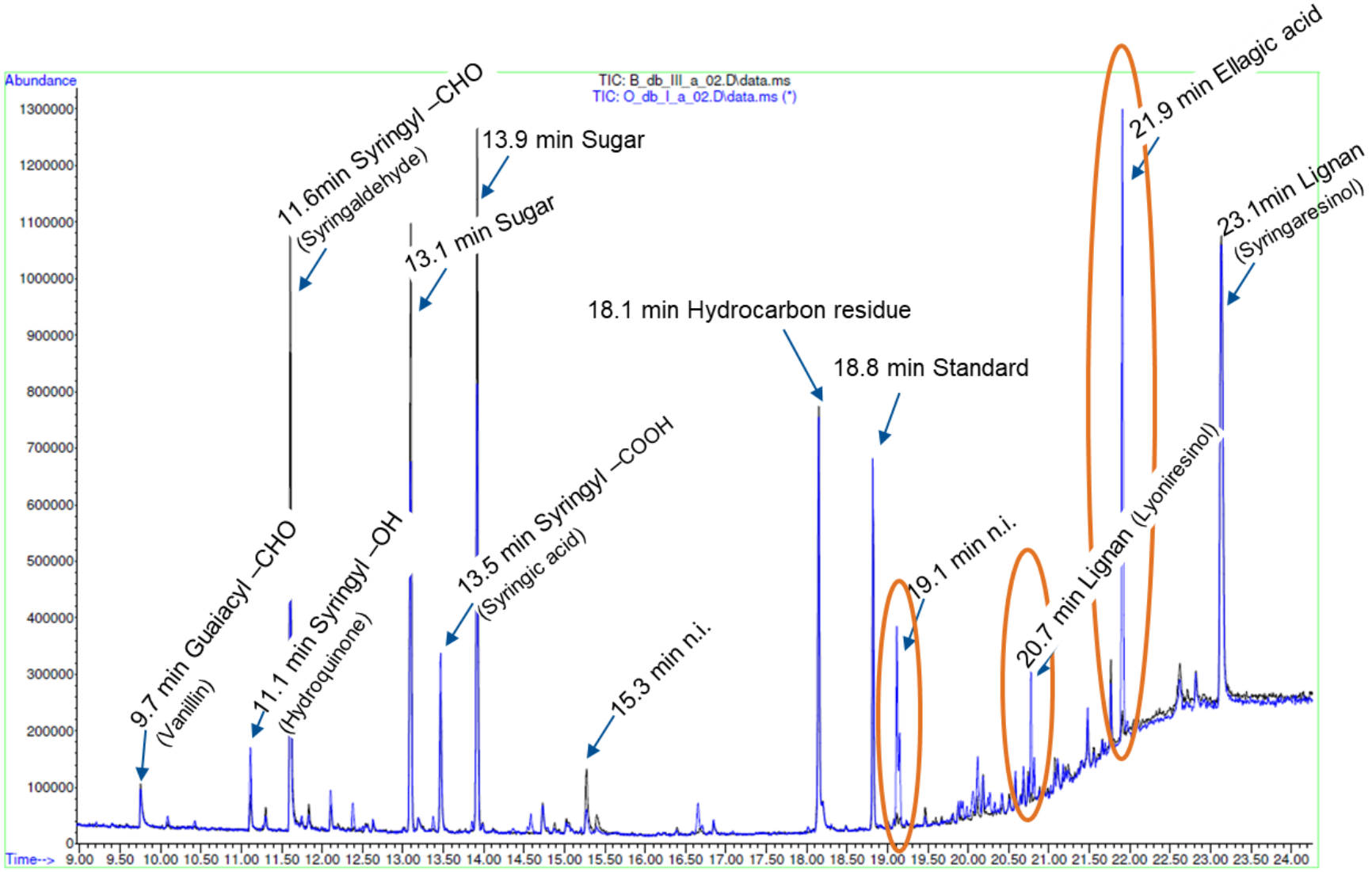

Figure 53: GC/MS chromatogram of SPE separated supernatant (eluate) of oak (blue) and beech db (black) after 48 h enzymatic hydrolysis without additives; orange circles mark peaks, which are present in oak, but not in beech; n.i. = not identified (Chapter 5.16).

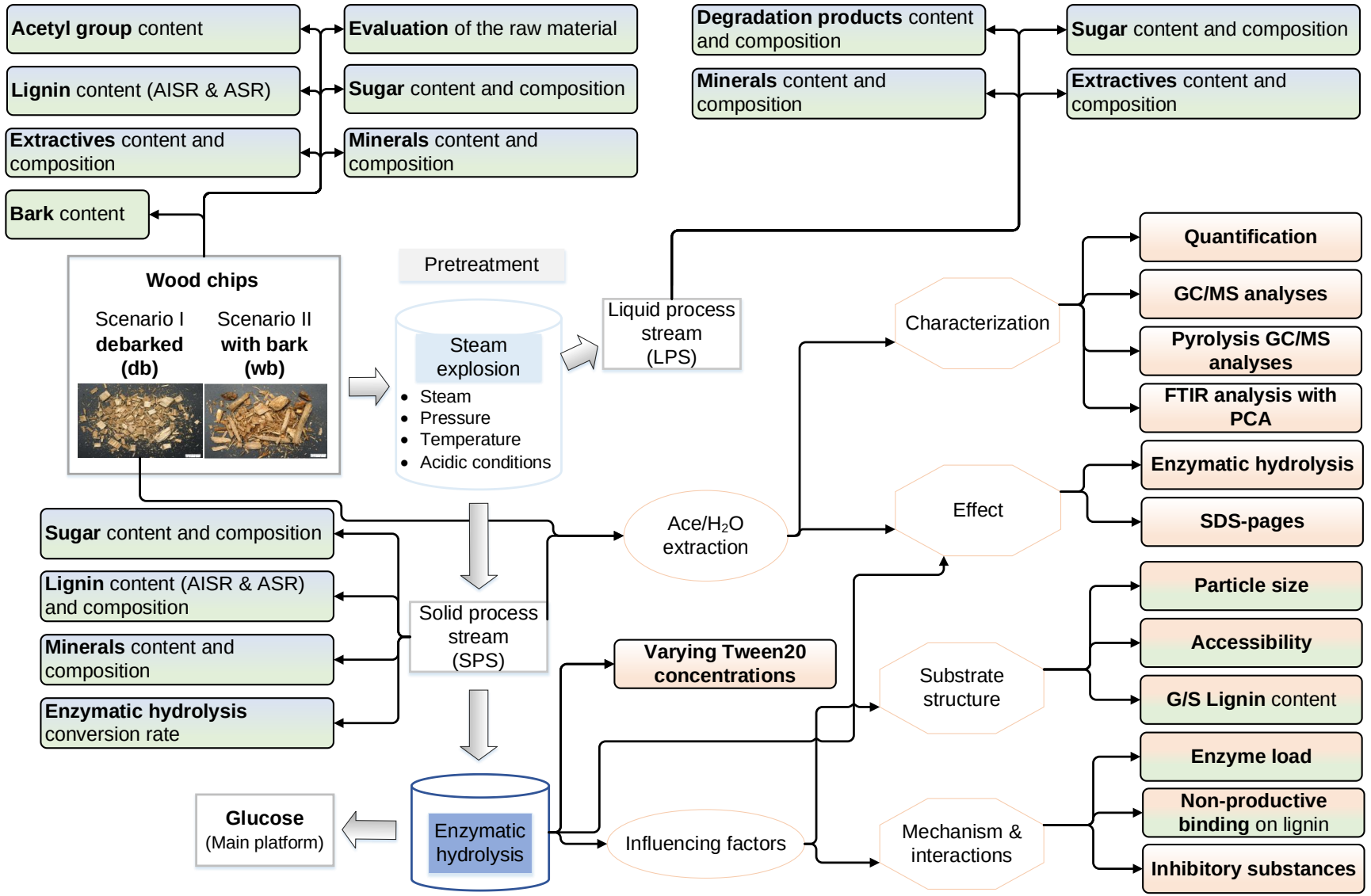

Figure 54: Frame of this thesis and analyses included in part 1 - 3, structured by the research questions (Chapter 0).