

Extraction and characterization of bark tannins from domestic softwood species

Dissertation with the aim of achieving a doctoral degree
at the Faculty of Mathematics, Informatics and Natural Sciences
Department of Biology
University of Hamburg

submitted by Sauro Bianchi
from Zurich (Switzerland)

Hamburg 2016

Evaluators of the dissertation:

Prof. Dr. Bodo Saake
University of Hamburg, Chemical Wood Technology
Hamburg, Germany

Prof. Dr. Frédéric Pichelin
Bern University of Applied Science, Institute for Materials and Wood Technology
Biel/Bienne, Switzerland

Date of the oral defense: 13th January 2017

Eidesstattliche Versicherung

Declaration on oath

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

Zurich, 12th September 2016

Sauro Bianchi



Acknowledgements

First, I wish to thank Silvia, who always lingered at my side and was unwittingly dragged into the deep secrets of tannins, arabinose and arabinans with me.

My warm thanks to Prof. Dr. Bodo Saake, from the University of Hamburg, for his patience, support and critical hints during my research. I really appreciated the opportunity to work under his supervision. With his clever, sharp, sagacious and humble points of view, he taught me more than just wood chemistry.

Many thanks to PD Dr. Gerald Koch, from Thünen Institute, who followed and guided me through the topochemistry of softwood bark. His valuable comments, knowledge and professional approach have provided a steady reference throughout this research.

My gratefulness to Dr. Ingo Mayer from the Bern University of Applied Science for his strong belief in softwood bark extractives that made this study real. I enjoyed working under his assistance, his full availability and the freedom in which he let me operate.

Thanks to Prof. Frédéric Pichelin, who offered me the opportunity to join the research group at the Bern University of Applied Science. His optimistic and proactive view to any new idea is a continuous incentive in my research.

Thank you to Dr. Ivana Krosiakova from the Zürich University of Applied Science for introducing me to the magic world of MALDI-TOF mass spectrometry. Her expertise and meticulous method permitted us to achieve astonishing results.

I cannot forget to thank Dr. Ron Janzon and Nicole Erasmy from the University of Hamburg, who patiently conducted me through the cleaving of bark polysaccharides, Daniela Paul from the Thünen Institute, for preparing all bark slides and instructing me in the UV-microspectrometry, and Prof. Florian Seebach and Matthias Knop from the University of Basel, for their kind availability in performing HPLC-MS measurements on my extracts.

My thanks to the Swiss National Research Program “Resource Wood” (NRP66) for their financial support on this project.

I extend my gratitude to the staff of the chemistry laboratory of the Institute for Materials and Wood Technology in Biel, and in particular to Christina Hinterleitner, whose help in setting up analytical methods, planning measurements and uncovering faults in existing procedures couldn't be summarized in a few lines. Thanks to Solene Barbotin for the perfect microtomies of SEM bark specimens, Regula Moser for compelling me to talk German and introducing the HPLC measurements, and Sandro Stuber for helping me in a never ending number of extractions and Folin-Ciocalteu assays.

Thank you to Rossella from “Il Giardino Segreto” in Pienza, where a large part of this thesis was written. It was our home far from home.

And thanks to all who convinced me that pursuing a doctorate at 40 years old was not foolish. I have no regrets.

Sauro Bianchi
Zurich, September 2016

List of papers and personal contribution

PAPER I.

Sauro Bianchi, Alexia N. Glöss, Ivana Kroslovakova, Ingo Mayer, Frédéric Pichelin.

Analysis of the structure of condensed tannins in water extracts from bark tissues of Norway spruce (*Picea abies* [Karst.]) and Silver fir (*Abies alba* [Mill.]) using MALDI-TOF mass spectrometry. *Industrial Crops and Products* 61 (2014): 430-437.

The author was the main contributor responsible for the interpretation of the MALDI-TOF spectra, both in the recognition of the possible monomeric units linked in the condensed tannin oligomers and in the identification of possible interferences due to co-extracted compounds (e.g. inorganic cations). He had primary responsibility for writing the article and is the corresponding author.

PAPER II.

Sauro Bianchi, Ivana Kroslovakova, Ron Janzon, Ingo Mayer, Bodo Saake, Frédéric Pichelin
Characterization of condensed tannins and carbohydrates in hot water bark extracts of European softwood species. *Phytochemistry* 120 (2015): 53-61.

The author planned, optimized and performed the extraction of softwood bark, the fractionation of the extracts and the thiolysis of the extracts and the corresponded fractions. He collaborated in the optimization of both the HPLC-UV method for the analysis of the thiolysed samples and MALDI-TOF mass spectrometry, and led the execution of the measurements. He performed the preparation and the hydrolysis of the samples for the carbohydrate analysis. He had primary responsibility for the interpretation of the data and writing the article, and he is the corresponding author.

PAPER III

Sauro Bianchi, Gerald Koch, Ron Janzon, Ingo Mayer, Bodo Saake, Frédéric Pichelin
Hot water extraction of Norway spruce (*Picea abies* [Karst.]) bark: analyses of the influence of bark aging and process parameters on the extract composition.
Holzforschung 70 (2016): 619-631

The author planned the experimental design for the analysis of the influence of the extraction process parameters and of the bark aging on the extraction yield and composition of the extracts. He performed the electronic microscopy observations and the

punctual measurements of UV absorption spectra on samples prepared by co-workers. He led the analysis of the extracts in the response surface method and performed the statistical analysis of the results. He executed the thiolysis followed by HPLC-UV of the extracts and also on powdered bark and performed the preparation and the hydrolysis of the samples for the carbohydrate analysis. He had primary responsibility for the interpretation of the data, writing the article and he is the corresponding author.

Author

Sauro Bianchi



Chairman of the supervising committee

Bodo Saake



UNIVERSITÄT HAMBURG
Zentrum Holzwissenschaft
Chemische Holztechnologie
Leuschnerstr. 91 B · D-21031 Hamburg

Summary

The bark of softwood species is a major by-product of the wood and pulp industry. The considerable presence in softwood bark of phenolic extractives and, in particular, of condensed tannins represents a potential for its valorization.

Condensed tannins are oligomers made of flavan-3-ol units. Besides their traditional use as tanning agents in the leather industry, they are regarded as natural sourced substituents of phenols in thermosetting resins (e.g. wood adhesives, foamed materials, composites), aldehyde scavengers, heavy-metal chelators, antioxidants and as bioactive additives. Nowadays, condensed tannins are mostly extracted from Black wattle bark (*Acacia mearnsii* [De Wild.]) and quebracho heartwood (*Schinopsis lorentzii* [Engl.] and *balansae* [Engl.]), both growing in the tropical or sub-tropical regions.

Extraction with hot water is the most common methodology for the recovery of tannins. When the process is applied to softwood bark was presented with two disadvantages in comparison to Quebracho and Black wattle extraction: the lower yield of extraction and the co-extraction of non-tannin compounds, mainly carbohydrates. The presence of such compounds impairs the extracts' properties (e.g. condensation rate with formaldehyde, viscosity of extract solutions). Furthermore, tannin chemical and physical properties are considerably influenced by the hydroxylation pattern of the flavan-3-ol units and their degree of polymerization. A detailed knowledge of the tannins molecular structure, the composition of the extracts and the influence of extraction parameters on the extract characteristics is important to identify potentials and limitations of European softwood bark as a source of condensed tannins.

The bark of Silver fir (*Abies alba* [Mill.]), European larch (*Larix decidua* [Mill.]), Norway spruce (*Picea abies* [Karst.]), Douglas fir (*Pseudotsuga menziesii* [Mirb.]) and Scots pine (*Pinus sylvestris* [L.]) were extracted with water at 60°C. The extracts were characterized according to their content of phenolic monomers, phenolic oligomers, condensed tannins, carbohydrates (mono-, oligo-, polysaccharides) and inorganic compounds (ashes). Condensed tannin structures were analyzed by MALDI-TOF MS and HPLC-UV after acid thiolysis. Composition of extracted carbohydrates was investigated by HPAEC after acid hydrolysis. Topochemistry of Norway spruce bark was analyzed by UV-microspectrometry. Total bark extractives and non-extractable tannins were determined by extraction with solvents of increasing polarities and HPLC-UV after direct thiolysis, respectively. Furthermore the changes in extract yield and extract composition during prolonged outdoor storage of Norway spruce logs and at the variation of the extraction process parameters were analyzed.

Total yields of hot water bark extractions were between 25.9 and 120.2 g/kg dry bark, which corresponded to about one third of total bark extractives. All extracts showed the

presence of phenolic monomers (5–24%), oligomers (16–45%), mono-/oligosaccharides (4–24%), polysaccharides (10–41%) and inorganic compounds (2–5%). The relative ratio between the different compound classes varied distinctively among the species. The highest phenolic oligomer concentration was detected in European larch, and the lowest in Scots pine. The extracted tannins were identified as procyanidins and prodelphinidins with an average polymerization degree between 3.5 and 6.7 units. Glycosylation and presence of stilbene monomeric units in the tannin oligomers were detected. Monosaccharides were mainly represented by glucose and fructose. Extracted polysaccharides were attributed to pectin such as arabinans, arabinogalactans, galacturonans and glucans. The constant presence of non-extractable condensed tannins in the bark was recognized. Their amount was 3 to 9 times higher than the extracted tannins, with the highest ratio corresponding to Scots pine. Prolonged storage of Norway spruce bark results in the leaching (or degradation) of phenolic monomers, mono-/oligosaccharides and a part of the tannins. The separation of tannins from mono-/oligosaccharides and phenolic monomers could be partially achieved through successive extractions at increasing temperatures from 30 to 90°C. Higher temperatures favored the extraction of pectins and the oxidation of tannins. The co-extraction of tannins and pectins could not be avoided within any temperature range.

The study highlighted that bark extracts from European softwoods species are characterized by a heterogeneous composition and complex condensed tannin structures, which clearly differentiate them from the currently used wattle and quebracho extracts. Their physical and chemical properties, e.g. condensation rate with aldehydes, are therefore expected to differ as well. The use of such extracts in tannin-based resins requires the tailoring of the existing formulations or the development of alternative solutions. The purification of softwood bark tannins from the large amount of co-extracted carbohydrates, of which additional costs will deter the commercial attractiveness of the product, should be carefully considered on a case-by-case basis. Alternatively, the exploitation of possible synergies between tannin and carbohydrates, e.g. co-reactions among them, are worth further investigations.

Zusammenfassung

Die Rinde von Nadelhölzern ist ein wichtiges Nebenprodukt der Holz- und Papierindustrie. Die in der Rinde enthaltenen phenolischen Extraktstoffe, insbesondere die kondensierten Tannine, besitzen ein Potential für eine stoffliche Verwertung mit erhöhter Wertschöpfung.

Kondensierte Tannine sind aus Flavan-3-ol-Einheiten aufgebaute Oligomere. Neben der traditionellen Nutzung als Gerbstoff in der Lederherstellung werden kondensierte Tannine als auf nachwachsenden Rohstoffen basierte Substitute von Phenolen in wärmehärtbaren Harzen (z.B. in Holzklebstoffen, geschäumten Materialien und Verbundwerkstoffen) oder als Aldehyd-Fänger, Schwermetall-Chelatbildner, Antioxidantien und bioaktive Additive eingesetzt. Die momentan kommerziell erhältlichen kondensierten Tannine werden primär aus Rinde der Schwarzholz-Akazie (*Acacia mearnsii* [De Wild.], Black wattle, Mimosa) und Kernholz von Quebracho (*Schinopsis lorentzii* [Engl.] und *Schinopsis balansae* [Engl.]) gewonnen, mit Verbreitungsgebieten in den Tropen und Subtropen.

Die Heißwasserextraktion ist dabei die am weitesten verbreitete Gewinnungsmethode für kondensierte Tannine aus Rinden. Die Heißwasserextraktion von Nadelholzrinden beinhaltet jedoch zwei Nachteile gegenüber der bei Schwarzholz-Akazie und Quebracho: eine niedrigere Extraktionsausbeute und die Co-Extraktion weiterer chemischer Verbindungen, vor allem von Kohlenhydraten. Diese zusätzlich extrahierten Verbindungen beeinträchtigen anwendungsrelevante Eigenschaften der Extrakte (z.B. Kondensation mit Formaldehyd, Viskosität von Lösungen). Die chemischen und physikalischen Eigenschaften der kondensierten Tannine werden hauptsächlich über das Hydroxylierungsmuster der Flavan-3-ol-Einheiten und den Polymerisationsgrad bestimmt. Eine genaue Kenntnis der molekularen Struktur der Tannine, der stofflichen Zusammensetzung der Extrakte und des Einflusses der Extraktionsparameter auf die Eigenschaften der Extrakte ist wichtig, um sowohl das Potential als auch die Einschränkungen der heimischen Nadelholzrinden als Rohstoff zur Gewinnung kondensierter Tannine beurteilen zu können.

Rinden der Weißtanne (*Abies alba* [Mill.]), Lärche (*Larix decidua* [Mill.]), Fichte (*Picea abies* [Karst.]), Douglasie (*Pseudotsuga menziesii* [Mirb.]) und Kiefer (*Pinus sylvestris* [L.]) wurden wässrig bei einer Temperatur von 60°C extrahiert. Die Extrakte wurden auf ihren Gehalt an phenolischen Monomeren, phenolischen Oligomeren, kondensierten Tanninen, Kohlenhydraten (Mono-, Oligo-, Polysacchariden) und anorganischen Verbindungen (Aschegehalt) untersucht. Die chemische Struktur der kondensierten Tannine wurde mittels

MALDI-TOF-MS und HPLC-UV nach saurer Hydrolyse untersucht. Die Zusammensetzung der extrahierten Kohlenhydrate wurde mittels HPAEC nach saurer Hydrolyse bestimmt. Topochemische Untersuchungen an Fichtenrinde wurden mittels Universalmikrospektralphotometrie (UMSP) durchgeführt. Der Gesamtextraktstoffgehalt sowie der Gehalt an nicht-extrahierbaren Tanninen in den Rinden wurde durch stufenweise Extraktion mit Lösemitteln aufsteigender Polarität und anschließender HPLC-UV nach saurer Hydrolyse bestimmt. Zudem wurden die Extraktionsausbeute und Zusammensetzung der Extrakte an Rindenproben von frei bewitterten Fichtenstämmen bei längerer Lagerdauer und die Einflüsse der Extraktionsparameter untersucht.

Die Ausbeute bei Heißwasserextraktion lag zwischen 25.9 und 120.2 g/kg Rindeatro, etwa ein Drittel der insgesamt in der Rinde vorliegenden Extraktstoffe. In allen Extrakten konnten phenolische Monomere (5-24%), phenolische Oligomere (16-45%), Mono- und Oligosaccharide (4-24%), Polysaccharide (10-41 %) und anorganische Verbindungen (2-5%) nachgewiesen werden. Das Mengenverhältnis der Stoffgruppen variierte zwischen den Baumarten erheblich. Die höchste Konzentration an phenolischen Oligomeren wurde für Rinde der Lärche, die niedrigste für Kiefernrinde gemessen. Die extrahierten Tannine konnten als Procyanidine und Prodelphinidine mit einem durchschnittlichen Polymerisationsgrad zwischen 3.5 und 6.7 charakterisiert werden. Auch Glycosylierungen und das Vorkommen von Stilben-Einheiten in den Tannin-Oligomeren konnten nachgewiesen werden. Glucose und Fructose waren die mengenmäßig am höchsten konzentrierten Monosaccharide. Bei den extrahierten Polysacchariden handelte es sich primär um Pektine, insbesondere Arabinane, Arabinogalaktane, Galakturonane und Glukane. Ein großer Teil der kondensierten Tannine im Rindengewebe konnte nicht extrahiert werden. Dieser Anteil lag drei- bis neunmal höher als der Anteil der extrahierbaren Tannine, mit den höchsten mengenmäßigen Unterschieden bei der Kiefernrinde. Die fortlaufende Lagerung frei bewitterter Fichtenstämmen führte zum Auswaschen oder Abbau der phenolischen Monomere, Mono-/Oligosaccharide und eines Teils der Tannine in der Rinde. Die schrittweise Extraktion bei steigenden Extraktionstemperaturen von 30 bis 90°C erwies sich als Möglichkeit, Tannine von Mono-/Oligosacchariden und phenolischen Monomeren ansatzweise zu trennen. Noch höhere Extraktionstemperaturen führten zu verstärkter Extraktion von Pektinen und zur Oxidation der Tannine. Eine zeitgleiche Extraktion von Tanninen und Pektinen fand allerdings bei jeder Extraktionstemperatur statt.

Im Rahmen der Arbeit konnte belegt werden, dass sich die Rindenextrakte europäischer Nadelhölzer in ihrer Zusammensetzung und der Komplexität der Struktur der kondensierten Tannine deutlich von den kommerziell erhältlichen Extrakten der Schwarzholz-Akazie und von Quebracho unterscheiden. Entsprechend können Unterschiede

in den chemischen und physikalischen Eigenschaften erwartet werden, z.B. für die Kondensationsrate mit Formaldehyd. Für Anwendungen in tanninbasierten Harzen erscheint deshalb eine Anpassung der Formulierungen oder die Entwicklung alternativer Lösungen als erforderlich. Eine mit hohen Zusatzkosten verbundene Aufreinigung der Tannine aus Nadelholzrinden durch Abtrennung der im großen Umfang mitextrahierten Kohlenhydrate dürfte eher nur in Einzelfällen in Frage kommen. Alternativ sollte eine gezielte Nutzung möglicher Synergien zwischen Tanninen und Kohlenhydraten, z.B. durch Co-Reaktion, weiter untersucht werden.

Table of contents

1. Introduction	1
1.1 <i>Background</i>	1
1.2 <i>Objectives</i>	3
2. Current state of knowledge	5
2.1 <i>Hydrolysable tannins</i>	5
2.2 <i>Condensed tannins</i>	6
2.2.1 Molecular structure	6
2.2.2 Distribution in the plant kingdom	8
2.2.3 Anatomical localization	9
2.2.4 Complexation with other plant constituents	10
2.2.5 Biological functions	11
2.2.6 Seasonal variations and aging	11
2.2.7 Structure and properties relationships	12
2.2.8 Commercial tannins	14
2.3 <i>Softwood bark</i>	15
2.3.1 Anatomy	15
2.3.2 Structural components of softwood bark	16
2.3.3 Non-structural components of softwood bark (extractives)	17
2.4 <i>Hot water extraction of softwood bark</i>	24
2.4.1 Hot water extract composition	24
2.4.2 Extraction process parameters	26
2.4.3 Addition of chemicals	26
2.4.4 External factors	27
2.4.5 Modification of condensed tannins during extraction	28
2.4.6 Post treatments of the extracts	29
2.4.7 Alternative extraction methods	30
2.4.8 Industrial extraction	30
3. Experimental	33
3.1 <i>Bark sampling</i>	33
3.2 <i>Bark topochemistry and composition</i>	34
3.3 <i>Bark extractions</i>	35
3.4 <i>Extract composition analysis</i>	35
3.5 <i>Condensed tannin characterization</i>	37

4. Results and discussion	39
4.1 <i>Softwood bark anatomy and chemistry</i>	39
4.1.1 Softwood bark anatomy	39
4.1.2 Softwood bark composition	39
4.1.3 Topochemistry of Norway spruce bark	43
4.2 <i>Softwood bark hot water extract characterization</i>	45
4.2.1 Total yield and extract composition	45
4.2.2 Phenolic monomers and other small molecular weight extractives	50
4.2.3 Molecular structure of the extracted condensed tannins	54
4.2.4 Monomeric composition of extracted carbohydrates	60
4.3 <i>Analysis and optimization of the softwood bark extraction process</i>	62
4.3.1 Extractions at different bark particle sizes	62
4.3.2 Response surfaces analysis of bark hot water extraction	64
4.3.3 Successive extractions at increasing temperature	68
4.3.4 Prolonged storage of bark exposed to natural weathering	72
5. Conclusions	79
6. Literature	83
7. Index of figures	93
8. Index of tables	95

Table of abbreviations used

A_{280}	UV-absorbance at 280 nm
AGP	arabinogalactan protein complex
Ara	arabinose
D_x	dispersity
ESI	electrospray ionization
Fru	fructose
Gal	galactose
GalA	galacturonic acid
Glc	glucose
HPAEC	high performance anion exchange chromatography
HPLC	high performance liquid chromatography
MALDI-TOF	matrix assisted laser desorption ionization – time of flight
Man	mannose
mDP	mean degree of polymerization
MS	mass spectrometry
MW	molecular weight
MW_N	number-average molecular weight
PAD	pulsed amperometric detector
PC	procyanidins
PD	prodelphinidins
Raf	raffinose
Rha	rhamnose
RT	retention time
SEC	size exclusion chromatography
SEM	scanning electronic microscopy
SPE	solid phase extraction
Suc	sucrose
UMSP	UV-microspectrometry
X_N	number-average degree of polymerization
Xyl	xylose

Table of cited plant species

Common name	Scientific name
Aleppo pine	<i>Pinus halepensis</i> [Miller]
Austrian pine	<i>Pinus nigra</i> [Arnold]
Balsam fir	<i>Abies balsamea</i> [Mill.]
Black wattle (or Mimosa)	<i>Acacia mearnsii</i> [De Wild.]
Chestnut	<i>Castanea sativa</i> [Mill.]
Dahurian larch	<i>Larix gmelinii</i> [Rupr.]
Douglas fir	<i>Pseudotsuga menziesii</i> [Mirb.]
Eastern hemlock	<i>Tsuga Canadensis</i> [L.]
Engelmann spruce	<i>Picea engelmannii</i> [Parry]
European larch	<i>Larix decidua</i> [Mill.]
Jack pine	<i>Pinus banksiana</i> [Lamb.]
Japanese larch	<i>Larix kaemperi</i> [Carr.]
Loblolly pine	<i>Pinus taeda</i> [L.]
Lodgepole pine	<i>Pinus contorta</i> [Douglas]
Maritime pine	<i>Pinus pinaster</i> [Aiton]
Mexican yellow pine	<i>Pinus oocarpa</i> [Schiede]
Norway spruce	<i>Picea abies</i> [Karst.]
Quebracho	<i>Schinopsis lorentzii</i> [Engl.] or <i>balansae</i> [Engl.]
Radiata pine	<i>Pinus radiata</i> [D.Don]
Scots pine	<i>Pinus sylvestris</i> [L.]
Shortleaf pine	<i>Pinus echinata</i> [Mill.]
Silver fir	<i>Abies alba</i> [Mill.]
Slash pine	<i>Pinus elliotii</i> [Engeln.]
Spotted mangrove	<i>Rhizophora stylosa</i> [Griff.]
Stone pine	<i>Pinus pinea</i> [L.]
Sumatra pine	<i>Pinus merkusii</i> [Jungh. & De Vriese]
Turkish pine	<i>Pinus brutia</i> [Tenore]
Virginia pine	<i>Pinus virginiana</i> [Mill.]

1. Introduction

1.1 Background

Bark represents between 10 and 20% of the total volume of wood plants. The annual bark yield from pulp and sawmills in North America and Europe is approximately 20 and 12 million tons, respectively (Feng et al. 2013; EUROSTAT 2015). Worldwide, the disposal of bark from the wood industry is estimated to be 160 million m³ (about 60 million tons), mostly derived from softwood species (Xing et al. 2006). Therefore, softwood bark represents a largely available biomass. The current consumption of bark is limited to energy production, through direct combustion in wood processing plants, and compost substrate in horticulture. Both solutions provide a limited added value to bark. Its poor mechanical properties and heterogeneous anatomical structure does not permit its efficient use for manufacturing of particleboards and fiberboards. A feasible option might be represented by the production of low-density insulation boards (Kain et al. 2014).

The considerable presence of extractives in bark tissues might represent an opportunity for the improvement of the bark value chain (Feng et al. 2013). In particular, softwood bark extracts have been shown to be rich in phenolic compounds, and especially in condensed tannins (Haslam 1989a; Porter 1989). The interest in plant extracts rich in tannins goes beyond their traditional use in the leather industry as tanning agents. The ability of condensed tannins to quickly condense with aldehydes and isocyanates (Pizzi 2008) to form chemically stable molecular networks has made them a suitable naturally sourced substitute for resin thermosetting formulations.

Wood adhesive thermosetting formulations based on the crosslinking between phenolic compounds and formaldehyde that have the phenolic part partially or completely substituted by bark extracts were developed in the 70s (Pizzi 1982a). The possibility of adhesive formulations using non-toxic aldehydes, formaldehyde-free crosslinkers (e.g. hexamethylenetetramine), or that exploit the autocondensation of tannins without the need of any added crosslinker, further focused the attention on bark extracts (Meikleham et al. 1994; Pizzi et al. 1995; Pizzi and Tekely 1995; Pichelin et al. 1999; Trosa and Pizzi 2001; Ballerini et al. 2005). Wood boards bonded with such tannin-based formulations showed lower formaldehyde emissions than the equivalent products containing standard urea-formaldehyde adhesives (Heinrich et al. 1996; Kim and Kim 2004; Pichelin et al. 2006). The suitability of tannin-rich bark extracts as natural substitutes in thermosetting resins is not limited to wood adhesives. Foamed material based on a mixture of condensed tannins and furfuryl alcohol were widely investigated and characterized (Tondi and Pizzi 2009;

Lacoste et al. 2015). These products showed noteworthy insulation and fire-resistance properties comparable to synthetic phenolic foams. Phenolic resins enriched with tannins were used as a matrix in composite materials containing sisal, flax or bagasse fibers (Barbosa et al. 2010; Sauget et al. 2013).

Besides thermosetting resins, condensed tannins resulted suitable compounds in several other applications. Their heavy metal chelation ability permits the use of softwood bark extracts as flocculants in wastewater treatments. Tannin-gels or microporous crumbs, produced through the reaction of the extracts with aldehydes, demonstrated metal removal efficiencies in contaminated waters close to or higher than activated carbons (Yamaguchi et al. 1992; Sanchez-Martin et al. 2011). Similar performances were observed in the direct use of milled softwood bark soaked in water, in some cases after pre-treatment with formaldehyde (Seki et al. 1997; Gaballah and Kilbertus 1998; Martin-Dupont et al. 2011; Liang et al. 2014). Corrosion inhibition properties were also observed in steel samples pre-treated with products based on softwood bark extracts (Ross and Francis 1978; Matamala et al. 2000). Biological antioxidant properties of phenolic monomers and oligomers extracted from softwood bark have been widely documented (Kähkönen et al. 1999; Packer et al. 1999; Pietarinen et al. 2006; Diouf et al. 2009). Pharmacological effects of condensed tannins such as antibacterial, antiviral, enzyme inhibitor, antimutagenic and antitumoral, next to some specific interactions with vascular and cardiac systems, were also reported (De Bruyne et al. 1999).

The suitability of the bark extracts in the various reported applications strongly depends on their actual composition and the molecular structure of the contained condensed tannins. For example, in the development of wood adhesives based on tannins, most of the formulations were originally developed using the extracts from Black wattle bark. The possibility to substitute Black wattle extracts in the formulations with softwood bark extracts was investigated (Dix and Marutzky 1987; Zhiquan et al. 1994; Yazaki and Collins 1994; Roffael et al. 2000; Valenzuela et al. 2012). Lower yields of condensation with formaldehyde, higher viscosities and shorter pot-life were observed. The dissimilar behaviors were assigned to the difference between the tannins extracted from the two botanical sources in the hydroxylation patterns and polymerization degree, as well as to the higher concentration of non-tannin compounds, and especially carbohydrates, in the softwood bark extracts (Pizzi 1982b; Yazaki 1985; Weissmann 1985b; Garnier et al. 2001). Similarly, while Black wattle and Turkish pine crude bark extracts could be efficiently used in the manufacturing of foamed materials, Norway spruce bark extracts had to be partially purified from the co-extracted carbohydrates in order to achieve comparable performances (Cop et al. 2015). The heavy metal chelating and corrosion inhibition properties also significantly differed among bark extracts from different species (Yoneda and Naktsubo 1998; Matamala et al. 2000). Tannins characterized from different chemical structures also

showed different selectivity in the precipitation of proteins, most likely due to the need for stereospecificity between tannin and protein molecules (Zucker 1983).

A detailed characterization of the composition of the softwood bark extracts and of the chemical structure of the extracted bark tannins is thus important to better understand the actual possibility of bark as a source of condensed tannins.

1.2 Objectives

With the partial exception of Norway spruce, no systematic study has so far been performed on the characterization of hot water extracts from the bark of Northern-Central European softwoods.

The main objectives of this research were therefore the characterization of the hot water bark extracts from European softwood species and the analysis of the extraction process through which they were obtained. For the study, the following five species were considered, which represent approximately 90% of the Northern-Central European softwood forest: Silver fir (*Abies alba* [Mill.]), European larch (*Larix Decidua* [Mill.]), Norway spruce (*Picea abies* [Karst.]), Douglas fir (*Pseudotsuga menziesii* [Mirb.]) and Scots pine (*Pinus sylvestris* [L.]).

Individual project aims were:

- Development of an analysis protocol for the determination of the composition of hot water extracts from the bark of softwood species, with a focus on condensed tannins and carbohydrates
- Evaluation of the influence of extraction parameters (e.g. temperature, time, particle size) and bark aging on the yield and extract composition, with a particular focus on the tannin to non-tannin compound ratio
- Identification of simple extraction strategies and likely applications of softwood bark extracts, also in comparison with currently used tannin-rich extracts (e.g. Black wattle)

2. Current state of knowledge

The term “extractives”, or secondary metabolites, is commonly used to label all the non-structural components of plant tissues. Several classes of compounds, with the common feature that they can all be extracted with a solvent from the biological matrix, are included in this expression, e.g. polyphenols, terpenoids, fats, waxes, organic acids, mono-, oligo- and polysaccharides, proteins, inorganic ions. The polyphenol class itself includes different compounds, e.g. phenolic acids, stilbenoids, lignans, coumarins, flavonoids and their respective glycosides and oligomers.

Tannins represent a group of polyphenolics almost ubiquitous in plant tissues. The actual composition of vegetable tannins has always been considered a problematic point in extractives chemistry, mostly due to their polymeric structure, the trickiness of their efficient isolation from other extractives, and the dispersity in their properties (Haslam 1989a). Two classes of chemical compounds are embraced under the same name “tannin”: hydrolysable tannins and condensed tannins. Their only shared property is the ability to form complexes with proteins and polysaccharides, which represents the chemistry behind the traditional silk dyeing and the leather tanning processes and contributes to tannin-containing foods and beverages having a typical astringent taste (Porter 1989; Haslam 1989a). Hydrolysable and condensed tannins are indeed two different chemical compounds, most likely with different functions in the plant metabolisms (Zucker 1983).

2.1 Hydrolysable tannins

The expression hydrolysable tannins covers a broad range of gallic acid esters that can be extracted from the bark, heartwood, leaves, fruits and galls of various plant species. The main sources are chestnut (*Castanea* spp.), myrabolans (*Terminalia* spp.), oak (*Quercus* spp.), sumac (*Rhus* spp.) and tara (*Caesalpinia* spp.) (Haslam 1989b; Pizzi 2008).

The most reported hydrolysable tannin is the penta-*O*-galloyl glucose (Figure 1a). Mono- and polygalloyl glucose esters with 2 to 12 galloyl units were also identified. Galloylester chains between 2 and 4 units linked to the central glucose are also common, like in the case of tannic acid (Figure 1b). The occurrence of a central quinic acid core in place of glucose was reported, in particular for tara and galls tannins (Clifford et al. 2007). All these compounds are generally labelled as gallotannins and are characterized by a disc-like molecular conformation (Zucker 1983; Haslam 1989a).

The oxidative coupling of vicinal galloyl ester groups, often followed by the opening of the pyranose ring and further coupling between galloyl groups, leads to the formation of ellagitannins, whose most known members are tellimagrandin, casuarictin (Figure 1c), potentillin, pedunculagin, castalin, castalagin (Figure 1d) and relative isomers. Different to gallotannins, ellagitannin molecules are nearly spherical in shape (Zucker 1983; Haslam 1989a). Oxidative coupling can also interest distinct pentagalloyl glucose units with the formation of dimers and trimers (Haslam 1989a). Pasch and Pizzi (2002) proposed that the major fraction of the hydrolysable tannin in living tissue is actually of a polymeric nature, and the extracted compounds are merely cleaved degradation products of *in situ* large molecules made of polygalloylglucose units.

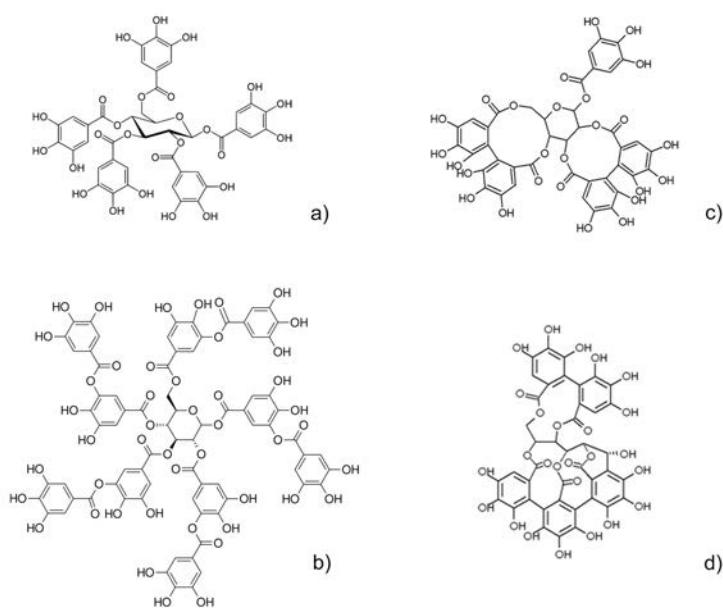


Figure 1: Schematic molecular structure of some compounds classified as hydrolysable tannins: a) pentagalloylglucose; b) tannic acid; c) casuarictin; d) castalagin.

2.2 Condensed tannins

2.2.1 Molecular structure

Condensed tannins, also known as proanthocyanidins, are oligomers made of polyhydroxyflavan-3-ol units, which can differ in their hydroxylation pattern (Figure 2). Their most common monomeric units are fisetinidol, robinetinidol, catechin, gallocatechin and their epimers, epicatechin and epigallocatechin (Porter 1992). The oligomers are generally made of homogenous monomeric units with the exception of the terminal monomer, which frequently show different hydroxylation and/or stereochemistry (Porter 1992; Pasch et al.

2001). In relation to the dominant units in the molecules, different types of condensed tannins are defined: profisetinidins, prorobinetinidins, procyanidins and prodelphinidins, which are oligomers mainly composed of fisetinidol, robinetinidol, catechin (or epicatechin) and gallocatechin (or epigallocatechin) units, respectively (Figure 2). A further classification of the condensed tannins is based on the hydroxylation pattern of the phenolic A-ring, which strongly influences their chemical properties (e.g. condensation rate with aldehydes, autocondensation, internal rearrangement). Procyanidins and prodelphinidins, which have a 5,7-dihydroxylated A-ring, are labelled as phloroglucinol tannins. Profisetinidins and prodelphinidins, which have a 7-mono-hydroxylated A-ring, are instead defined as resorcinol or 5-dehydroxylated tannins (Figure 2). Acylation and glycosylation of the flavan-3-ol units with mono- and oligosaccharides were reported, in particular for phloroglucinol tannins (Porter 1989; Ishimaru et al. 1991).

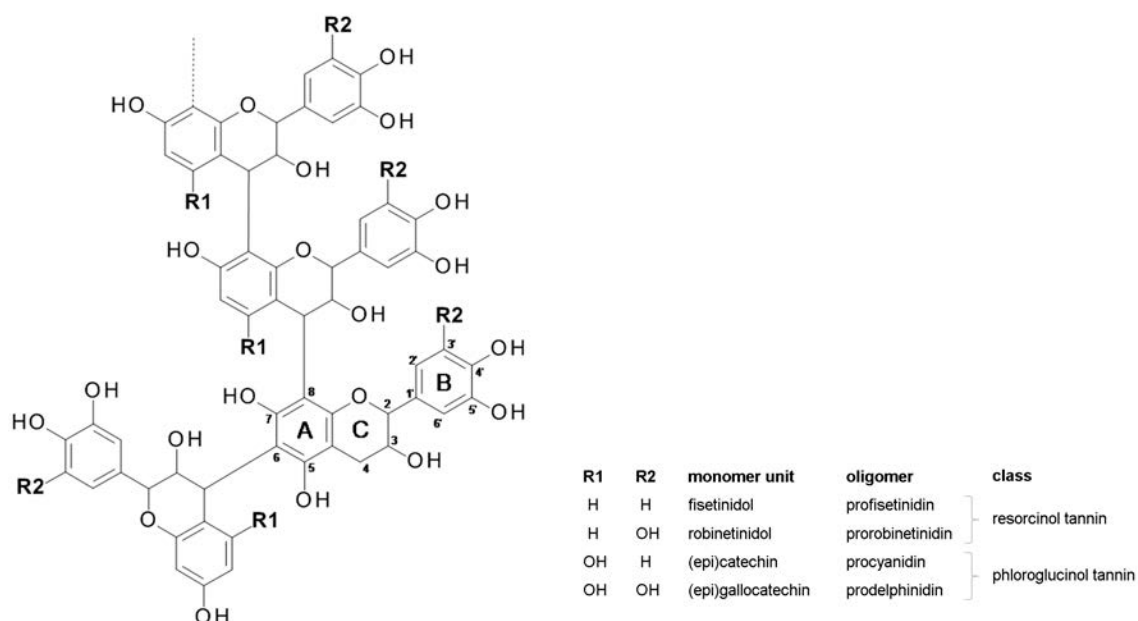


Figure 2: Schematic molecular structure of condensed tannins

The link between the monomers is mostly constructed through C4-C8 bonds (Figure 2) forming linear structures in a helical conformation. Right- or left-hand helixes are both likely, as a function of the stereochemistry in position C3 of the heterogeneous ring (Zucker 1983). Side-branching through C4-C6 bonds were observed (Porter 1989). In particular, resorcinol tannins typically show the presence of a phloroglucinol unit bonded at both the C8 and C6 positions with other resorcinol units forming “angular” tannins (Figure 2) as they were named after Botha et al. (1978). The occurrence of dimers having, in addition to the C4-C8

bond, a C2-O-C7 ether link (A-type proanthocyanidins) was also reported mainly in relation to auto-oxidative processes (Porter 1992).

The number-average degree of polymerization (X_N) of condensed tannins can be actually measured only on the extracted compounds, and therefore it varies, not only according to the botanical source, but also with the used extraction procedure. Phloroglucinol tannins showed X_N between 6 and 15 (Porter 1992). Resorcinol tannins are generally shorter, with X_N between 3 and 10 (Porter 1992, Venter et al. 2012a). High dispersity (D_x) of the extracted tannins was generally detected. Trimers and tetramers are considered the majority of the extractable oligomers, and the occurrence of longer oligomers rapidly decreases as their degree of polymerization increases (Porter 1992). The likelihood of tannin molecules made of several hundred units was nevertheless suggested. The existence of two distinct maxima in the polymerization degree distribution curve was also reported and attributed to the presence of two different metabolic classes of phenolic oligomers (Porter 1989).

In their basic structure of pure flavan-3-ol oligomers, condensed tannins are colorless with a maximum absorption wavelength between 270 and 280 nm as a result of the dominant degree of hydroxylation of the phenolic B-ring (Hümmer and Schreier 2008). Oxidation during aging of the plant tissues might develop conjugated carboxyl and carbon-carbon double bonds, which also absorb in the visible range between 400 and 600 nm (Stafford 1988; Porter 1992).

2.2.2 Distribution in the plant kingdom

Condensed tannins are widely distributed in the plant kingdom and have been detected in a large variety of tissues, e.g. xylem, bark, fruits, seeds, leaves, roots and galls. A chemiotaxonomy of plant species based on the presence and typology of condensed tannins was suggested by Bate-Smith (Haslam 1989a). The ability to synthesize tannins is predominantly confined to woody families. Herbaceous families scarcely present such capacity, or at a considerably lower yield. Since woody families are more primitive than herbaceous, the loss in tannin biosynthesis capability can be regarded as a trend in the botanical evolution. Another evolutionary trend was observed in the elimination or loss in functionality (e.g. through methylation) of the hydroxyl groups in flavonoids (Harborne 1977). Phloroglucinol tannins are almost ubiquitous in woody plants, but are the only tannins detected in the evolutionary primitive softwood species. Resorcinol tannins, which show a 5-deoxy A-ring, are almost confined to the relatively advanced Leguminosae and Anarcadiaceae species (Porter 1992). Propelargonidins, which are phloroglucinol tannins having a 4'-monohydroxylated B-ring, are absent from some of the most primitive species such as Magnoliidae, Caryophylladae and Hamamelidae (Porter 1989).

2.2.3 Anatomical localization

It is generally assumed that polyphenolic extractives are deposited in the protoplast of cells and practically absent from the cell walls (Parameswaran et al. 1976; Zucker 1983; Nunes et al. 1999; Krekling et al. 2000). Their synthesis takes place in specialized cells (polyphenolic cells) that have been developed by differentiation of standard parenchyma cells (Krekling et al. 2000). The polyphenols appear in the endoplasm of polyphenolic cells as small vesicles that coalesce forming larger vacuoles (Figure 3). Eventually, these could grow until to fill up the whole inner volume of the cells (Toscano Underwood and Pearce 1991).

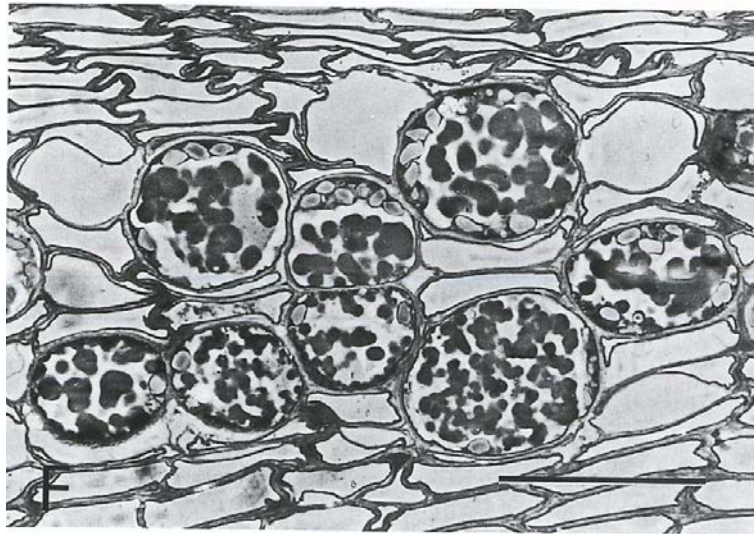


Figure 3: Parenchyma cells (4 years old) in the secondary phloem of Norway spruce. The dark deposits in their protoplast indicate the presence of polyphenolics (courtesy of Krekling et al. 2000). Bar = 50 μ m.

The penetration in the cell walls of phenolic monomers and small oligomers is considered likely, but limited to dead tissues (e.g. heartwood, outer bark) after destruction of the lipid membrane (tonoplast) that enclosed the polyphenolic vacuole (Hillis 1985). The presence of phenolic oligomers infiltrated in the inner surface of cell walls was observed close to fungal checks (Stafford 1988) and in phellem of beech bark (Prislan et al. 2012).

Within a homogeneous tissue, polyphenolic cells are generally randomly distributed (Stafford 1988). In the secondary phloem of Norway spruce and Stone pine multiple circumferential layers of polyphenolic cells were observed, suggesting the development of a polyphenolic cell layer at the beginning of each growing season (Krekling et al. 2000; Nunes et al. 1999). Enhanced production of polyphenolic deposits was observed in the area surrounding necrotic spots after fungal or insect attack (Stafford 1988). The leakage of polyphenolic deposits from polyphenolic cells to the lumen of adjacent non-polyphenolic death-cells was detected after development of tyloses (Hillis 1985).

2.2.4 Complexation with other plant constituents

The complexation with proteins and carbohydrates is the most known feature of both hydrolysable and condensed tannins (Haslam 1989a). The principal binding mechanisms are hydrogen and hydrophobic interactions, while covalent and ionic bonds are doubtful (Zucker 1983; Porter 1989).

The quick development of tannin-protein complexes is the cause of the astringent taste of tannin-rich foods and beverages (e.g. tea, wine). Once formed, the complexes become generally insoluble and precipitate in aqueous solutions (Hagerman 1989). The presence of a polyhydroxylated aromatic ring having an *ortho*-conformation, e.g. the galloyl groups in hydrolysable tannins and the flavanol B-ring in condensed tannin, facilitates complexation with proteins (Zucker 1983).

Complexes between condensed tannins and polysaccharides rapidly develop as well. During thin-layer chromatography, condensed tannins originally dissolved in methanol could not be released after being applied on the cellulose-based substrate (Stafford 1988). However, this behavior wasn't observed with hydrolysable tannins, suggesting their reduced ability to form complexes with cellulose (Zucker 1983).

Tannin-polysaccharide complexes can be very strong and stable. Unsuccessful efforts in the chromatographic separation of tannins and polysaccharides extracted from Spotted mangrove leaves and Dahurian larch bark, initially led to a misinterpretation of these complexes as covalently bonded copolymers (Porter 1989). The molecular conformation of the polysaccharides is a key factor for the stability of the complex, which are more likely formed in the presence of crevices, bowls and hollows with size comparable to that of a phenolic ring (Ya et al. 1989). The presence of *in situ* complexes between tannins and polysaccharides or proteins is considered among the main causes for the presence of non-extractable compounds in plant tissues (Stafford 1988; Porter 1989).

An alternative interpretation of the limited solubility of tannins is the presence of complexes with metal ions (e.g. Fe^{3+} , Ca^{2+}) that are formed by *ortho*-configured polyphenols. The metal ions act as a bridge among tannin oligomers resulting in high molecular weight sets of molecules with limited solubility. The complexes could be formed both *in situ* and during extraction, forming a precipitate that can be re-dissolved only if the pH of the extract is reduced (Yazaki 1987).

Matthews et al. (1997a) proposed that the non-extractable fraction of tannins isn't merely related to complexes with other plant constituents but also to the development of weak chemical linkages (e.g. hydrogen bonds, π - π interactions) among tannin oligomers. In a study on the interaction between salivary proline-rich proteins and tannins, Murray et al. (1994) demonstrated that tannin-tannin complexes are stronger than tannin-peptide complexes.

2.2.5 Biological functions

The biological roles of tannins in plants predominantly concern protection against herbivores and parasites and the inhibition of litter degradation. Both functions have been attributed to the strong and almost irreversible complexation of tannins with cellulose, pectins and proteins (Porter 1989; Haslam 1989a). The sequestration of iron ions through complexation might also be a complementary mechanism in the protection function of tannins (Scalbert et al. 1999). After complexation, the ions remain unavailable to the herbivore that ingested the plant tissue, which is then regarded as an unattractive food source. Zucker (1983) suggested differences in the biological roles of hydrolysable and condensed tannins, with condensed tannins being more specifically “designed” against microbes and pathogens, thus blocking or strongly inhibiting the accessibility of degrading enzymes to starch, cellulose, pectins and structural proteins. Hydrolysable tannins, however, are more active against herbivorous insects and vertebrates, blocking the digestive proteins in their gastric and intestinal systems.

The slower decay of bark than wood tissue in litter was attributed to the higher concentration of tannins and other polyphenols in bark than in wood (Ganjegunte et al. 2004; Vane et al. 2006). Slowly degradable tannin-protein complexes play an important role in the rate regulation of the nitrogen release in soil.

A structural role of tannins was suggested by Stafford (1988) in the case of decayed cells. The infiltration of high molecular weight phenolics in the cell walls might partially counteract the lost bearing ability of the degraded cell. Tannins have also been associated with allelopathy. The release of tannins in the soil from foliage litter inhibits the seed germination of antagonist species, or likewise controls the excessive proliferation of new sprouts of the same species (Porter 1989).

2.2.6 Seasonal variations and aging

Changes in the tannin concentration and molecular structure were reported during both the annual growing cycle and the tissue aging. The total phenolic extraction yield showed in general a minimum in the early spring and a maximum in autumn (Porter 1989; Tiarks et al. 1992). The decrease of extractable phenolics during the winter dormancy was mainly related to their leaching or transformation without replacement of new deposits (Tiarks et al. 1992). The extraction yield of stilbene glucosides from Norway spruce bark throughout the year followed a comparable path (Solhaug 1990). Seasonal changes in the phenolic deposits of Norway spruce inner bark were observed by light microscopy (Krekling et al. 2000). From August to January, the deposits in the vacuoles showed a progressive increase in the opacity and the development of a dense grainy structure. At the beginning of the new growing season (April/May), the phenolic density was considerably lower and

remained almost constant throughout the summer. Since this tissue had not been extensively exposed to the rain, leaching could have only had a minor role, thus, intrinsic biological and chemical modifications of the deposited phenolics were supposed.

The biological decay of condensed tannins is considered a slow process, unlike hydrolysable tannins, which are readily decomposed in glucose and gallic acid by the *tannase* enzyme (Field and Lettinga 1992). A partial degradation of condensed tannins was observed only after an abundant supply of sugars, but limited to the smallest tannin oligomers. After being cleaved, flavanol monomers are mostly transformed in phenolic acids, e.g. protocatechuic acid. The formation of carboxycyclohexane was also considered likely (Field and Lettinga 1992). In needle or leaf litter a quick leaching of the shorter oligomers (less than 4 units) was reported, followed, during prolonged outdoor storage, by a slower and progressive decrease of the observable polymerization degree and an increase of glycosylation (Schofield 1998; Maie et al. 2003; Zhou et al. 2012).

The most common reactions during aging of condensed tannins are oxidative coupling and internal isomerization. The coupling of flavan-3-ol monomers through native *oxidase* enzymes was observed in oak bark (Porter 1989). The same reaction was also considered likely between the flavan-3-ol units of condensed tannins (Porter 1992). The rearrangement of profisetinidins and procyanidins with the formation of A-type procyanidins, phlobatannins and catechinic acid (Figure 4) was observed in alkaline media (Steenkamp et al. 1985; Burger et al. 1990; Bae et al. 1994; Hashida et al. 2003). These isomerizations are thought to also occur naturally *in situ* with the facilitation of specific enzymes. The consequence of the progressive rearrangement and coupling of the tannins is the development of water insoluble compounds called phlobaphenes (Foo and Karchesy 1989a). The actual nature of phlobaphenes is not yet clear. Their chemical structure is most probably related to those of condensed tannins, however evidence of additional functional groups was reported. The proposed structure of phlobaphenes in Douglas fir bark was a composite polymer made of procyanidins, flavonoid monomers (e.g. taxifolin), lignans, and mono-/oligosaccharides, most likely as glycosyls (Foo and Karchesy 1989a).

2.2.7 Structure and properties relationships

The chemical and physical properties of condensed tannins are strongly dependent on the type of monomeric units and the degree of polymerization. The hydroxylation pattern of the A-ring in the flavan-3-ol units influences the reactivity of tannins towards aldehydes, the most exploited reaction in the formulation of tannin-based resins. Phloroglucinol tannin solutions showed gelling times in presence of formaldehyde almost 10 times shorter than solution with resorcinol tannins (Pizzi and Stephanou 1994b). Differences in the condensation rate with formaldehyde have been identified between the stereoisomers

catechin and epicatechin (Takagaki et al. 2000). The hydroxylation at the B-ring is more important in correlation to the metal chelating properties. Tannins with trihydroxylated B-rings showed almost double the scavenging capacity than those with a dihydroxylated pattern (Yoneda and Nakatusbo 1998).

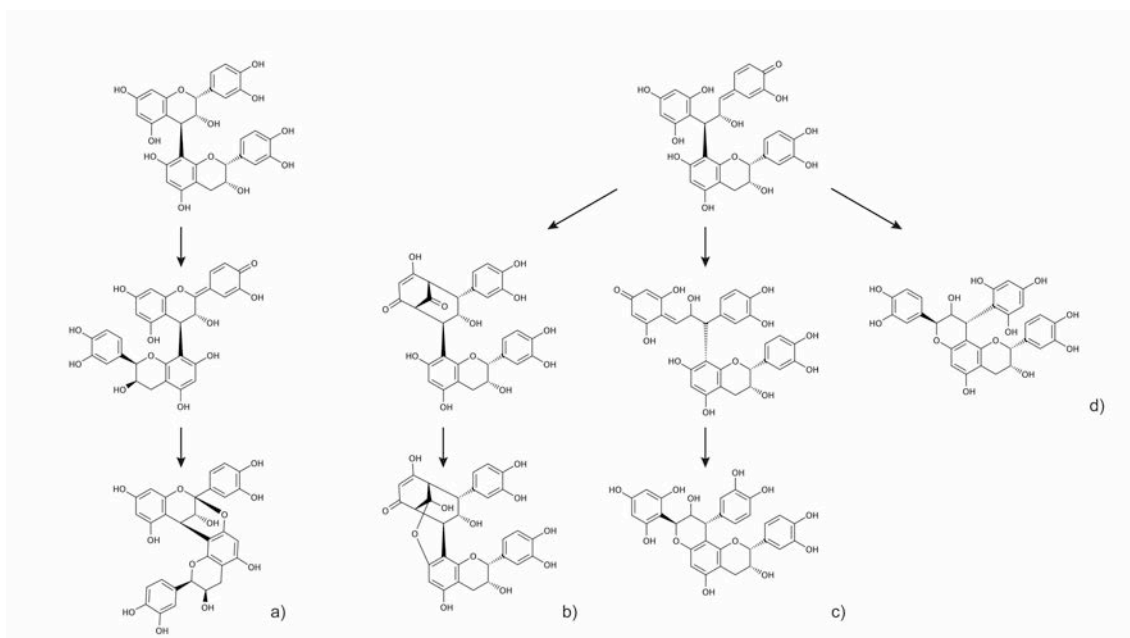


Figure 4: Most typical pyran ring rearrangements of a procyanidin dimer leading to the formation of a) A-type procyanidins, b) catechic acid dimer, c,d) phlobatannins (modified from Burger et al. 1990 and Hashida et al. 2003).

With increasing degree of tannin polymerization, higher viscosities, enhanced heavy metal chelation and better antioxidant properties were observed (Weissmann 1981; Yazaki and Hillis 1980; Yoneda and Nakatsubo 1998; Garnier et al. 2001; Jerez et al. 2007b). The length and flexibility of the oligomers were identified as the main factors that control the complexation of tannins with carbohydrates and proteins (Ya et al. 1989).

The need for a mutual molecular structural affinity between tannin and proteins or tannin and carbohydrates was proposed by Zucker (1983) as a key condition for the formation of complexes, but contradictory results have so far been reported. Hagerman (1989) reported an enhancement in the protein precipitation capability at increasing degree of hydroxylation and in the presence of flavan-3-*O*-galloyl esters. However, in more recent works, tannins that differed in the stereoconfiguration of the monomeric units, hydroxylation and polymerization degree showed only elusive differences in the protein binding ability (Kraus et al. 2003; Norris et al. 2011).

The different responses of various tannins to standard chemical assays such as Folin-Ciocalteu, vanillin and acid butanol, was related to their different chemical structures

(Appel et al. 2001; Schofield et al. 2001; Kraus et al. 2003; Norris et al. 2011). However, a clear dependence of the assay resulting from the tannin conformational characteristics has not yet been defined. For example, Kraus et al. (2003) showed higher Folin-Ciocalteu assay yields in proportion to increasing trihydroxylated B-rings and *cis*-configurations (e.g. epicatechin, epigallocatechin) in the monomeric units, but Norris et al. (2011) didn't confirm such dependencies.

The stability of the interflavonoid bond is dependent on the hydroxylation of the A-ring of the monomeric unit. Phloroglucinol tannins can be easily depolymerized by acid cleavage in the presence of nucleophiles such as cysteamine, phenylmethanethiol, phloroglucinol or sulfite anions (Hemingway 1989). Resorcinol-tannins can be depolymerized only by hydrogenolysis at very low temperatures (Steynberg et al. 1994). Linkages between monomeric units different from flavan-3-ols are also resistant to acid cleavage: the breakage of the covalent bond between flavanols and stilbene units detected in Sitka spruce bark tannins was possible only through catalytic hydrogenation (Matthews et al. 1997b). Linear C4-C8 interflavonoid linkages are more easily cleaved than C4-C6 bonds of the branched oligomers, and slight differences were detected between equatorial (e.g. C4 α -C8) and axial (e.g. C4 β -C8) linkages (Hemingway and McGraw 1983).

A higher susceptibility to *in situ* oxidative degradation of prodelphinidins than procyanidins was suggested from observations on Radiata pine bark (Porter 1989). Younger bark tissues contained similar amounts of uncolored procyanidins and prodelphinidins, while older tissues were clearly reddish (most likely due to the presence of oxidized products) and contained only procyanidins.

2.2.8 Commercial tannins

The most traded condensed tannins are extracted from the bark of Black wattle and the heartwood of quebracho. Their combined yearly production is about 200,000 tons, mainly located in Argentina, Brazil, India, South Africa, Tanzania and Zimbabwe (Porter 1989; Pizzi 2008). Both extracts contain resorcinol tannins and, more specifically, fisetinidins for quebracho and robinetinidins for wattle (Pizzi and Stephanou 1994b; Pasch et al. 2001; Venter et al. 2012a, 2012b). Their X_n was reported between 3.2 and 6.7 (Garnier et al. 2001; Venter et al., 2012a), but the existence of oligomers with polymerization degree up to 10 units was shown by MALDI-TOF mass spectrometry (Pasch et al. 2001).

Phloroglucinol tannins were industrially extracted from the bark of Radiata pine in Chile and New Zealand during the 90s, but production has since ended. They were mostly used in adhesive formulations for particleboards and fiberboards (Valenzuela et al. 2012). Highly purified extracts from the bark of Maritime pine, mostly composed of procyanidins, are traded under the name Pycnogenol[®] for pharmaceutical preparations.

Extracts from Eastern hemlock, Pecan nut, Peanut and Gambier leaves, rich in phloroglucinol tannins, were traded in the past, but their availability on the current market is almost completely lacking.

2.3 Softwood bark

2.3.1 Anatomy

The bark is the lignocellulosic tissue situated externally and peripherally to the vascular cambium of woody plants. Its anatomical structure presents clear peculiarities compared to wood. Two distinct regions can be recognized: inner bark (or secondary phloem) and outer bark (or primary phloem).

The inner bark develops from the same cambial tissue of xylem and its thickness ranges from 0.5 to 15 mm, depending on the plant species. Thin walled parenchyma cells represent the majority of inner bark, of which only the most internal (newer) layers are active. Annual growth rings are difficult to recognize because of the irregular and often collapsed arrangement of the parenchyma cells. Specific cell types, not existing in wood, can be observed in the inner bark, namely sieve cells and sclereids (or stone cells). Sieve cells are longitudinally elongated elements that serve as lanes for the downward transportation of sap. Sclereids are thick, irregularly shaped and highly lignified cells, which occur in isolation or in clusters among parenchyma cells (Howard 1971).

The outer bark is the most external tissue of a stem and can be over 100 mm in thickness. It is generated by the phellogen, a tangential oriented layer of meristematic cells, which have been developed by differentiation of the inner bark parenchyma cells (Figure 5c). Distinctive cell types are produced radiating inward and outward of the phellogen. The ingoing tissue is called phellogen and it is made of parenchyma cells (Figure 5a,d). Within the phellogen, the cells closer to the phellogen show an almost regular shape, thick walls, and are evenly arranged. At increasing distance from the phellogen, towards the inner bark, the cells become irregular, greatly expanded and with thinner walls (Howard 1971). At the outside of the phellogen a clearly recognizable tissue called phellem develops. The phellem is mostly made of thick walled and highly lignified cells arranged in multiple layers (Figure 5b). Parameswaran et al. (1976), highlighting the similarity of these cells with the inner bark sclereids, proposed the term "sclerotic phelloid" to describe this tissue. External to the sclerotic phelloid, a few layers of highly suberized, thin walled and flattened cork cells are usually observed. The combination of phellogen, phellogen and phellem represent the periderm (Howard 1971). As the tree stem grows and expands, the initial periderm fractures and the inner bark produces a new phellogen. This, in turn, forms a new, innermost

periderm. Successive and discontinuous periderm bands, corresponding to the successive tissue development phases, are observable in the older bark tissues, producing the known scaled pattern of softwood bark (Nunes et al. 1999).

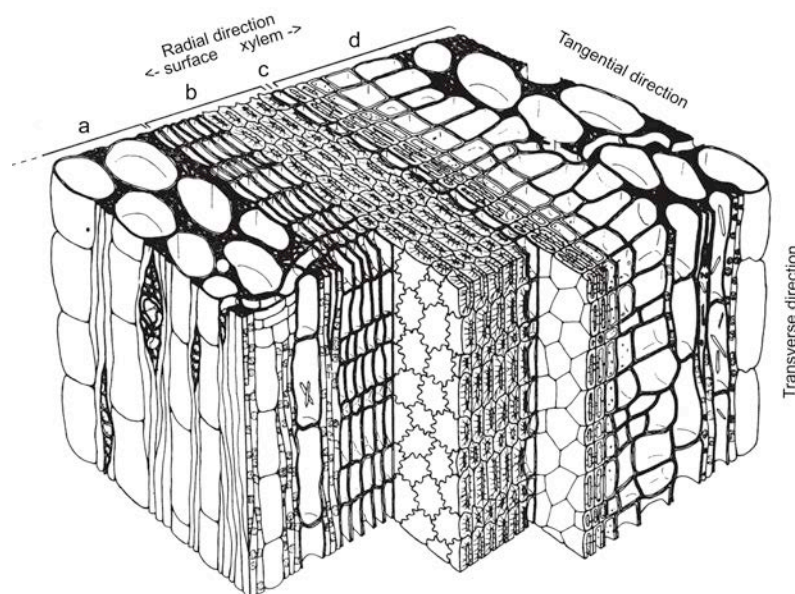


Figure 5: Schematic structure of softwood outer bark. Along the radial direction are the recognizable different periderm layers: a) older phelloderm; b) phellem; c) phellogen; d) younger phelloderm (modified from Howard 1971).

2.3.2 Structural components of softwood bark

The main components of bark, similar to wood, are polysaccharides and lignin. Cellulose (as glucose residue after hydrolysis of the bark) represents between 20% to 37% w/w of softwood bark (Timell 1961; Dietrich 1978; Fradinho et al. 2002; Valentin et al. 2010; Krogell et al. 2012). The cellulose concentration generally decreases moving from the inner towards the outer tissues (Fengel and Wegener 1983b; Krogell et al. 2012). Compared to the wood cellulose, the bark cellulose shows a lower polymerization degree and higher dispersity (Fengel and Wegener 1983b).

Hemicellulose represents between 9% and 26% w/w of softwood bark and, conversely to cellulose, its concentration increases moving from the inner towards the more peripheral tissues (Fengel and Wegener 1983; Krogell et al. 2012). The composition of bark hemicellulose consists of both structural (non-soluble) polysaccharides and extractable pectins. Structural polysaccharides have been identified in galactoglucomannans, glucomannans and arabino-4-*O*-methylglucuronoxylans. Their monomeric composition slightly differs across softwood species (Timell 1961; Dietrich et al. 1978; Valentin et al. 2010; Krogell et al. 2012).

Pectins represent probably the largest fraction of the softwood bark hemicellulose (Krogell et al. 2012; Miranda et al. 2012; Le Normand et al. 2014). In xylem tissues pectins were mostly localized in the middle lamella, in the primary cell walls and in the innermost S3 cell wall layers (Evert 2006; Altaner et al. 2010). Since the concentration of pectin in bark is more than three times greater than in xylem (Krogell et al. 2012), a broader diffusion of them throughout the cells is expected. Pectins, as bark extractives, will be further discussed in section 2.3.3D.

Lignin, determined by the Klason method after removal of the phenolic extractives, ranges between 13% to 33% w/w of the softwood bark (Dietrichs et al. 1978; Weissman 1985a; Fradinho et al. 2002; Valentin et al. 2010; Miranda et al. 2012). In Norway spruce, the increase of the lignin concentration moving from the inner to the outer bark was detected (Krogell et al. 2012). Guaiacylpropane and, to a more limited extent, p-hydroxyphenylpropane units were detected as the main monomeric units (Fengel and Wegener 1983; Fradinho et al. 2002). Zhang and Gellersted (2008), on the basis of 2D-NMR analysis, suggested that the actual amount of pure lignin in Norway spruce bark is limited to merely 3% w/w in the inner bark and 10% w/w in the outer bark. Klason-lignin might include in fact compounds characterized by a hybrid tannin-lignin structure that could be extracted through prolonged rinsing with aqueous acetone.

2.3.3 Non-structural components of softwood bark (extractives)

Softwood bark contains a considerable amount of extractives of various types. Successive extractions at increasing solvent polarity are usually performed to assess the total amount and the types of extractives. Non-polar compounds are firstly extracted using petroleum ether, diethylether, hexane or benzene. Ethanol, methanol, acetone (or an aqueous solution of them) are effective in the recovery of phenolic monomers, oligomers (e.g. tannins) and, partially, pectins. A fractionation between phenolic monomers and oligomers could be performed using first ethylacetate, in which the oligomers are less soluble than the monomers, and subsequently aqueous methanol or acetone. A following hot water extraction enables the recovery of the longest phenolic oligomers, but together with a considerable amount of pectins. A final treatment in hot alkaline water can extract the less soluble pectins (Fengel and Wegener 1983; Porter 1989).

The total yield of extraction and the relative composition of the bark extracts (Table 1) noticeably varied across softwood species, but also among analyses on the same bark performed by different researchers. This evidence highlights that a comparison of the extractive composition between different species can only be performed if consistent extraction and analysis protocols are used.

A. Non-polar extractives

Nonpolar extractives are mostly represented by waxes, fatty acids, lipids, resin acids, phytosterols, and terpenes. With few exceptions, they generally account for less than 50 g/kg of dry bark (g/kg_{DB}) (Table 1). Fatty and resin acids (e.g. oleic acid, linoleic acid, behenic acid, isopimaric acid, dehydroabietic acid) have been reported as the main non-polar extractives in softwood bark, with substantial variations in their relative amounts across species (Fengel and Wegener 1983; Sakai 2001; Krogell et al. 2012; Valentin et al. 2010).

B. Phenolic monomers

Phenolic monomer extractives include a large variety of compounds, e.g. flavonoids, stilbenes, lignans and their glycosides.

The flavanone taxifolin (Figure 6a), or dihydroquercetin, is the most frequently detected in softwood bark. The abundance of taxifolin in Douglas fir bark has been documented (Barton and Gartner 1958) and its occurrence in Norway spruce and various pine species has also been recently reported (Yazaki and Hillis 1977; Weissmann 1981; Pan 1995; Karonen et al. 2004; Yesil-Celitkas et al. 2009a/b; Jerez et al. 2009a; Ucar et al. 2013). In combination with taxifolin, taxifolin-3'-glucoside (Figure 6b) was usually detected. Taxifolin 4- and 7-glucosides (Figure 6c) were detected in traces in Norway spruce, Douglas fir and Scots pine (Hergert 1958; Foo and Karchesy 1989b; Pan 1995).

The flavanols kaempferol and quercetin (Figure 6d) are common softwood bark extractives that generally exist in combination with their glycosides. In particular, kaempferol-7-glucoside (Figure 6e) and kaempferol-3-rhamnoside (Figure 6f) were reported for Douglas fir bark (Yazaki and Hillis 1977; Foo and Karchesy 1989b; Pan 1995).

Free flavanols like catechin and galocatechin and, less frequently, their epimers epicatechin and epigallocatechin occur in most of the softwood bark in small amounts, most likely as by-products of condensed tannin biosynthesis (Sakai 2001; Yesil-Celitkas 2009a; Karonen et al. 2004). Glucosyls of catechin and epicatechin in position 7 (Figure 6g), 3' and 4' were found in Douglas fir, Norway spruce and some pine species (Foo and Karchesy 1989b; Pan 1995; Ucar et al. 2013). 3'-methylcatechin and its 7-glucoside were identified in Scots pine, Norway spruce and Douglas fir (Pan 1995; Foo and Karchesy 1989b). The presence of catechin-3-gallate (Figure 6h) and catechin-3-ferulate (Figure 6i) was reported for Austrian pine bark (Yesil-Celitkas 2009b).

The stilbene-3-glucosides astringin, isorhapontin and piceid (Figure 6j,k,l) are typical phenolic extractives from the bark of spruce species (Weissmann 1981; Solhaug 1990; Toscano Underwood and Pearce 1991; Mannila and Talvitie 1992; Pan 1995; Zhang and Gellerstedt, 2009; Krogell et al. 2012; Latva-Mäenpää et al. 2013). Their respective aglycones piceatannol, isorhapontingenin and resveratrol were also identified in spruce bark at smaller amounts. Piceatannol was found in Radiata pine bark, together with its

4'-glucoside, which differ from astringin by the glucosylation position (Yazaki and Hillis 1977; Jerez et al. 2009a). Similarly, a resveratrol-4'-glucoside was detected in Scots pine, together with its monomethyl derivative (Pan 1995). Pinosylvin monomethylether was identified in the acetone extract of Scots pine (Valentin et al. 2010). Stilbene glucoside dimers, double linked through a C-C and a C-O-C bond, were detected in Norway spruce bark extracts (Li et al. 2008; Zhang and Gellerstedt 2008).

Table 1 Extraction yields (g/kg_{DB}) of successive extractions of softwood barks with solvents at increasing polarity. The symbol “♦” indicates that no extraction was performed with the correspondent solvent (data from Dietrich et al. 1978; Labosky 1979; Weissmann 1981, 1983, 1985a; Laks 1991; Vazquez Penas et al. 1992; Sakai 2001; Fradinho et al. 2002; Valentin et al. 2010; Miranda et al. 2012).

Species	Extraction solvent						Total
	Hexane	Benzene	Ether	Ethanol	Acetone	Hot water	
Aleppo pine	♦	♦	25	100–208	♦	86–98	211–331
Austrian pine	♦	♦	26	31	♦	39	96
Balsam fir	♦	132	♦	33	♦	27	192
Douglas fir	♦	♦	54–90	112–169	♦	30–46	196–305
Eastern hemlock	♦	28	♦	219	♦	33	280
Engelmann spruce	♦	52	♦	259	♦	109	420
European larch	♦	♦	45	149	♦	90	284
Jack pine	♦	80	♦	124	♦	30	234
Japanese larch	♦	♦	37	284	♦	180	501
Loblolly pine	17	13	13	20	♦	19	82
Lodgepole pine	♦	287	♦	109	♦	56	452
Maritime pine	♦	♦	31	103	♦	32	166
	♦	22–34	8–10	105–126	♦	20–66	155–236
Mexican yellow pine	♦	♦	30	163	♦	71	264
	♦	66	05	29	♦	140	240
Norway spruce	♦	♦	47–93	56–198	♦	112–151	216–403
	♦	♦	24–78	♦	70–176	119–150	265–343
Radiata pine	27	10	29	♦	238	63	366
	♦	♦	41	120	♦	53	214
	15	15	40	50	♦	130	250
Scots pine	♦	♦	42–46	12–52	♦	48–92	106–188
	♦	63	10	18	♦	129	220
	♦	♦	♦	♦	56	137	193
Shortleaf pine	26	12	11	44	♦	20	113
Slash pine	21	20	12	73	♦	33	159
Sumatra pine	♦	♦	17	42	♦	73	132
Turkish pine	♦	50	♦	257	♦	178	485
Virginia pine	15	10	10	35	♦	19	89

Total stilbenes' concentrations in Norway spruce bark were reported between 5 and 100 g/kg_{DB} (Solhaug 1990; Mannila and Talvitie 1992; Krogell et al. 2012). This wide range is related to the noteworthy variations in the stilbenes' extraction yields observed both within the bark thickness and along the stem. A radial increase of stilbene concentration from the cambium to the living periderm was detected (Toscano Underwood and Pearce 1991), but diminished almost completely in the outer bark layers (Zhang and Gellerstedt 2008; Krogell et al. 2012). Yazaki and Hillis (1977) reported similar observations for the distribution of the piceatannol-4'-glucoside in *Radiata* pine bark, while its aglycone was found only in the outermost bark. Along the stem, higher concentrations of stilbenes were detected in proximity of the first branches than at the stump (Toscano Underwood and Pearce 1991). These data suggest that stilbenes are mostly located in young tissues and practically absent in the oldest bark, most likely because of degradation or modification during aging.

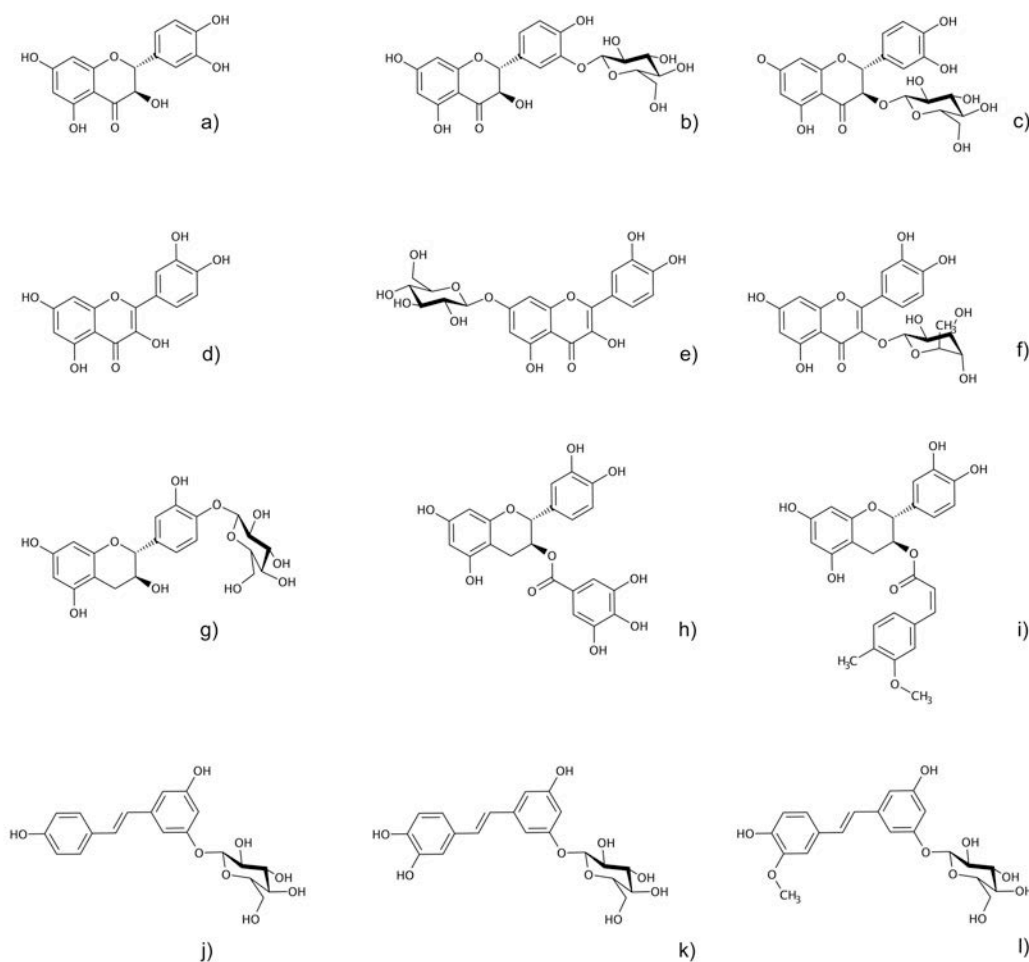


Figure 6: Molecular structure of some of the most common phenolic monomers detected in softwood barks. a) taxifolin, b) taxifolin-3'-glucoside, c) taxifolin-3-glucoside, d) kaempferol, e) kaempferol-7-glucoside, f) kaempferol-3-rhamnoside, g) catechin-4-glucoside, h) catechin-3-gallate, i) catechin-3-ferulate, j) piceid, k) astringin, l) isorhapontin.

A variation of the concentration of taxifolin within the bark was also detected (Yazaki and Hillis 1977). The compound was in fact detected only in the most external layers of the outer bark but it was completely missing in the inner tissues.

Lignans like taxiresinol, secoisolariciresinol, lariciresinol, pinobatol were identified in the bark of Scots pine and Silver fir (Pan 1995; Benkovic et al. 2014; Sinkkonen et al. 2006). Phenolic acids (e.g. protocatechuic, p-hydroxybenzoic, vanillic, p-coumaric acids) were also detected in Silver fir, Maritime pine, Radiata pine and Scots pine (Mun and Ku 2006; Jerez et al. 2009a; Benkovic et al. 2014).

C. Condensed tannins

The condensed tannins extracted from the softwood bark have been mostly identified in procyanidins (Porter 1989; Matthew et al. 1997a). Prodelphinidins have been detected in only a few species (e.g. Momi fir, Sakhalin fir, Slash pine and Lodgepole pine) and always in the presence of procyanidins (Porter 1989). Peculiar cases are Radiata and Patual pine. The inner (younger) periderm of these species contains a mixture of procyanidins and prodelphinidins, while outer (older) periderm have almost exclusively procyanidins (Porter 1989). This observation contrasts with the analysis of Longleaf pine bark extracts, for which an increased concentration of prodelphinidins was observed moving outward through the bark thickness (Porter 1989).

The monomeric units have predominantly a *cis*-configuration at the position C2 (epicatechin and epigallocatechin) (Porter 1989; Matthews et al. 1997a). An exception is represented by Radiata pine procyanidins, which presented a majority of *trans*-configured units (catechin and galocatechin) (Porter 1989; Matthews et al. 1997a; Jerez et al. 2009a).

Extracted softwood bark tannins showed X_n between 3 and 10 (Weissman 1981; Porter 1989; Matthews et al. 1997a). Dispersity D_x ranged from 2 to 7, indicating a broad distribution of the molecular size (Weissman 1981; Matthews et al. 1997a). Scots pine bark extracts analyzed by normal phase high performance liquid chromatography (HPLC) and electrospray ionization (ESI) mass spectroscopy showed the presence of procyanidins from dimers through to decamers and higher oligomers. Procyanidins longer than 10 units appeared to represent the larger weight fraction of the extracted tannins (Karonen et al. 2004; Hellström and Mattila 2008). Foo and Karchesy finely characterized procyanidins extracted from Douglas fir bark using ^{13}C - and ^1H -NMR (Foo and Karchesy 1989c, 1989d, 1991). Linear oligomers with C4-C8 interflavanol bonds were generally identified, but the occurrence of branching through C4-C6 bonds was observed at a relatively high frequency. Extension units were almost exclusively represented by epicatechin, while both catechin and epicatechin were identified as terminal units. Maritime and Radiata pine tannins were analyzed through thiolysis, ESI and MALDI-TOF mass spectrometry by Jerez and co-workers (Jerez et al. 2007a, 2007b, 2009a, 2009b). Maritime pine bark water extract showed to

contain procyanidins with $X_N = 5.3$, but the presence of oligomers up to 22 units was detected. Procyanidins in Radiata pine showed a higher average polymerization ($X_N = 7.6$), but the longest observed oligomer had 15 units. Navarrete et al. (2010) analyzed Maritime pine bark extracts by MALDI-TOF mass spectrometry and detected a frequent occurrence of gallate esters along the procyanidins backbone, which is not evident in the mass spectra recorded by Jerez et al. (2009b).

The presence of monomeric units different from flavan-3-ols in softwood bark tannins has been occasionally reported. Tannin oligomers extracted from the bark of Norway spruce were analyzed using 2D-NMR by Zhang and Gellerstedt (2008), which estimated that procyanidin units represented merely 53% of the tannins. The remaining units were represented by stilbene-derived structures (33%) and methoxylated lignin-related blocks (14%). The presence of stilbene glucosides in the molecular structure of Sitka spruce tannins was also detected by Matthews et al. (1997b) after hydrogenolysis. Tannins extracted from Turkish pine bark were identified as procyanidins with occurrences of glucosides and dihydroxybenzoate esters of flavanol units (Porter, 1989; Ucar et al. 2013).

The total amount of tannins in softwood barks is estimated between 50 and 370 g/kg_{DB} (Fengel and Wegener 1983). However, the determination of the actual amount of tannins in bark is tricky. The results are dependent on the extraction process and the analytical method used for the estimation of the tannins. An explicatory case is the quantification of tannins in Norway spruce bark presented in different studies that used different analytical protocols: total tannin amount ranged between 17 and 107 g/kg_{DB} (Matthews et al. 1997a; Zhang and Gellerstedt 2008; Krogell et al. 2012; Kempainen et al. 2014). A possible explanation of the dispersion of the results is the presence of condensed tannins in the bark tissues that can be extracted, and thus quantified, only in particular conditions (Stafford 1988). These compounds have been attributed to tannins blocked in plant tissues due to their large size (e.g. after the oxidative coupling of tannin oligomers) and the development of secondary bonds with other plant constituents or among themselves. The native chemical structure of tannins might also be responsible for their limited solubility, as was shown for tannins formed in pure protoplast cell culture preparations of Douglas fir (Stafford and Cheng 1980). Matthews et al. (1997a) performed acid thiolysis on softwood bark residuals after aqueous methanol extraction and detected a considerable further release of flavanol units that were attributed to non-extractable tannins. In Scots pine the amount of non-extractable compounds was almost comparable to the amount of those extracted. Using milder thiolysis conditions, Hellström and Mattila (2008) determined that the amount of non-extractable tannins in Scots pine bark was merely 5% of those extracted. Zhang and Gellerstedt (2008) performed prolonged extraction with acetone on Norway spruce outer bark that had already been extracted with a series of solvents at increasing polarity (total extraction yield = 225 g/kg_{DB}). A further 68 g/kg_{DB} of extractives were obtained with this

procedure. The extracted compounds were identified in procyanidins and were determined to be largely copolymerized with stilbenes and lignin-related blocks.

Higher amounts of tannins have been generally reported for the outer bark (Yazaki and Hillis 1977; Fengel and Wegener 1983; Zhang and Gellerstedt 2008). Krogell et al. (2012) reported the opposite trend in Norway spruce. The same trend was observed by Matthews et al. (1997a) for Scots pine and Austrian pine bark. The decrease in the extractable tannins moving from the inner to the outer bark was correlated with an increase of the non-extractable tannins in the same direction, suggesting a transition from extractable to non-extractable compounds during tissue aging. Likewise, an increase of the tannin polymerization degree from the inner towards the outer layers of Radiata pine bark was reported (Yazaki and Hillis 1977).

D. Carbohydrates

Mono-/oligosaccharides, starch and pectins represent an important fraction of softwood bark extractives. Sucrose, fructose, glucose and galactose (in descending order of concentration) were detected in hot water extracts of Norway spruce bark (Weissman 1984; Krogell et al. 2012). Their total concentration was between 30 and 48 g/kg_{DB}.

Pectins could be extracted with neutral or mild alkaline water. Extraction yields from 14 to 235 g/kg_{DB} were reported, mostly as a result of the temperature of extraction (Krogell et al. 2012; Le Normand et al. 2012). They are mainly associated with arabinans, arabinogalactans, glucans (callose), galactoxyloglucans, and polygalacturonans (Fu et al. 1972; Fu and Timell 1972a; Valentin et al. 2010; Krogell et al. 2012; Le Normand et al. 2012; Kempainen et al. 2014). Softwood bark arabinans were described as highly branched polymers with number-average molecular weight (MW_N) higher than 10⁴ Da (Figure 7 - Fu and Timell 1972a; Le Normand et al. 2012). The presence of rhamnogalacturonans branched with large arabinans in Norway spruce inner bark was proposed by Le Normand et al. (2014). In plant tissues, arabinogalactans occur often as arabinogalactan-protein (AGP) complexes, which are very soluble, diffusible and distributed in both the cell walls and the intercellular spaces (Evert 2006).

Starch (1.1 - 17.3 g/kg_{DB}) was determined and observed as granular bodies in the lumen of bark parenchyma cells at the end of the growing season (Dietrich 1978; Krekling et al. 2000; Kempainen et al. 2014; Le Normand et al. 2012).

E. Inorganic compounds

Inorganic ions such as calcium, potassium and, at minor concentrations, magnesium, manganese and phosphorous are common constituents of softwood barks (20 - 40 g/kg_{DB}) (Saarela 2005; Krogell et al. 2012; Miranda et al. 2012; Kempainen et al. 2014). Calcium, the most abundant ion, generally exists as insoluble calcium oxalate crystals. Their presence

within bark parenchyma cells was evidenced by electronic microscopy (Parameswaran et al. 1976; Nunes et al. 1999).

A seasonal variation in the concentration of potassium ions was observed, with a maximum and a minimum occurring during summer and at the beginning of winter, respectively (Krizaj 1996). Other elements like iron, boron, zinc, aluminum, barium and sodium were also detected at concentrations lower than 0.1 g/kg_{DB}.

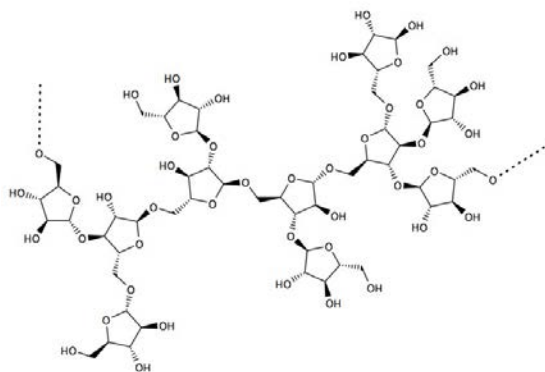


Figure 7: Schematic structure of a softwood bark arabinan (based on Fu and Timell, 1972a).

F. Other extractives

The storage of proteins in the bark of several softwoods, up to a concentration of 7.4 g/kg_{DB} for the case of Scots pine, was reported (Dietrich 1978; Wetzell and Greenwood 1989). Formic and acetic acids (21 g/kg_{DB}) were detected in Norway spruce bark extracts (Krogell et al. 2012), but their provenience was unsure and likely associated to *in vivo* degradation of the sample. Yazaki and Aung (1988) peculiarly reported the presence of aliphatic dicarboxylic acids in alkaline extracts from Radiata pine. These data are yet to be confirmed or disproved by other studies.

2.4 Hot water extraction of softwood bark

2.4.1 Hot water extract composition

The more efficient media for the extraction of tannins from softwood bark are acetone:water solutions between 70:30 and 90:10 v/v (Foo and Porter 1980; Stafford 1988). An organic solvent extraction is however considered unsuitable for a large-scale industrial process, and the use of hot water is generally preferred. The main disadvantage of hot water extractions is the low selectivity, with the consequent simultaneous extraction of both phenolic and non-phenolic compounds. The result is therefore a dilution of the actual

concentration of tannins in the extracts. The presence of polysaccharides in bark extract is of concern. They have been associated with the high viscosity of extract solutions (Weissman 1985b; Garnier et al. 2001) and waning bonding performance of tannin-based adhesives (Pizzi 1982a). Colloidal or other second order interactions between tannin oligomers and polysaccharides have been supposed.

Water extractions at 100°C of European larch bark showed a total extraction equal to 160 g/kg_{DB} (Weissman 1985a). Carbohydrates represented 35% w/w of the extracts. Methanolysis of the carbohydrate fraction showed a monomeric composition dominated by galactose, arabinose and polygalacturonic acid. The presence of pectic polysaccharides (e.g. arabinans, galactan, arabinogalactan and galacturonans) in the extracts was thus indicated. Size exclusion chromatography (SEC) of the phenolic fraction indicated the presence of oligomers from 2 to 20 units and the peculiar presence of four local maxima. The concurrent presence of different classes of phenolic oligomers, each of them with a distinct weight distribution curve, was therefore supposed.

Radiata pine bark extracted in the same conditions (Weissmann 1985b) resulted in a lower amount of carbohydrates (15% w/w). The monomeric composition of the extracted polysaccharides further indicated the presence of pectins, but with a noticeable lower fraction of arabinans. Size fractionation of the Radiata pine extracts by ultrafiltration showed that compounds with molecular weight lower than 10³, between 10³ and 10⁶, and above 10⁶ Da (as Dextran equivalents) represent 37.6%, 49.0% and 13.4% w/w of the extracts, respectively (Yazaki and Hillis 1985). The middle range fraction gave the maximum amount of precipitates when treated with formaldehyde (Stiasny number), indicating that most of the phenolic compounds were in this weight interval. In the lowest and highest mass fractions monosaccharides and gums were respectively collected.

Extraction of Norway spruce bark in boiling water gave a total yield of 110 g/kg_{DB}. Approximately 50% of the extract was represented by phenolic compounds (determined by gravimetry after preparative chromatography) and 18% by carbohydrates (Liiri et al. 1982; Tisler et al. 1986). SEC of the phenolic fraction indicated $MW_N = 602$ Da and $D_x = 6.2$. The high D_x suggested that phenolic compounds were characterized by a wide range of polymerization degrees, from monomer through to oligomers and higher polymers. More recently, Kempainen et al. (2014) performed a bench scale extraction of Norway spruce bark in water at 90°C, and obtained a total yield equal to 205 g/kg_{DB}. The extract was composed of approximately 45% tannins (determined by acid butanol assay), 30% polysaccharides (arabinans, galactans, galacturonans) and 7.5% free monosaccharides (prevalently glucose). The considerable differences in both the extraction yield and extract composition between the two studies on Norway spruce, which used quite similar extraction conditions, highlights the need for a better understanding of the influence of the extraction parameters on the extract characteristics.

2.4.2 Extraction process parameters

The increase of the extraction temperature was identified as the most effective factor to gain higher yields in the water extraction of softwood bark (Jorge et al. 1999; Vázquez et al. 2001; König and Roffael 2003; Kempainen et al. 2014). Extraction temperatures over 80°C showed an upsurge of the co-extracted carbohydrates and an increased concentration of mannose and xylose in the monomeric composition of the carbohydrate fraction (Fradinho et al. 2002). A degradation of the structural hemicellulose of the bark (galactoglucomannans, glucomannans, arabino-4-*O*-methylglucuronxylans) at these extraction temperatures was hinted.

The extraction time has a considerable influence up to 1 hour. However, for longer times no actual improvements were observed, especially in the case of low liquid:bark ratios (Yazaki 1985; Kempainen et al. 2014).

Extractions were generally performed using liquid:bark ratios between 5:1 and 12:1, with better extraction yields at the lowest values (Vázquez Penas et al. 1992; Jorge et al. 1999; Vázquez et al. 2001). Kempainen et al. (2014) performed extraction of Norway spruce bark at 20:1, 10:1 and 7:1 at different times and temperatures. For an extraction time up to 15 minutes, higher yields at the lower liquid:bark ratios were observed, in agreement with the other studies, however for longer extraction times (up to 2 hours), the highest extraction yields were observed for the highest liquid:bark ratio.

Fine milling to achieve an average particle size below 0.5 mm resulted in a noteworthy increase of the extraction yields, most likely due to diffusion limitations of the extractives through the bark tissue (Vázquez et al. 2001). Yazaki (1985) showed that the effect of the bark particle size is significant only for a pure water or slightly alkaline extraction. At strong alkaline conditions no appreciable differences were observed at the reduction of the particle size. Analysis on different size fractions of milled bark showed slightly different chemical compositions (Vázquez et al. 2001; Miranda et al. 2012). The finest particles (< 0.2 mm) showed less lignin and higher amounts of extractives as phenolics, carbohydrates and inorganic compounds. These variations correlated to the heterogeneity of bark, since various cell tissues were differently ground during milling.

2.4.3 Addition of chemicals

An increase of the total extraction yield from softwood bark was reported after addition of sodium hydroxide, carbonate, sulfite or bisulfite to the extraction water. Sodium hydroxide addition, which induced mild to strong alkaline conditions during the extraction, showed total extraction yields 4 to 10 times higher than those obtained in pure water (Liiri et al. 1982; Chen and Pan 1991). The huge increase in total yield often did not correlate to a

significantly higher phenolic yield, but mostly to an increased concentration of non-phenolic compounds in the extracts (Chen and Pan 1991; Yazaki 1985; Chupin et al. 2013).

The non-phenolic fraction has two distinct sources: the favored cleavage and extraction of hemicellulose fragments at alkaline conditions, and the residual sodium hydroxide in the dry extract. The most efficient extractions, in terms of Stiasny number of extracts, were performed at $\text{pH} < 10$, corresponding to a concentration of sodium hydroxide in the extraction water lower than 1% w/v (Dix and Marutzky 1983; Yazaki 1985).

Addition of sodium sulfite and/or bisulfite improved the tannin extraction yield, increased the solubility of the extracts and reduced their viscosity (Yazaki and Hillis 1980; Foo et al. 1983; Chen and Pan 1991; Sealy-Fisher and Pizzi 1992; Bertaud et al. 2012). The gain in the total extraction yield was less marked than in alkaline extractions, most likely due to less severe degradation of the bark hemicellulose. Kempainen et al. (2014) showed an improvement of the tannin:polysaccharide ratio in Norway spruce bark extracts by adding 2.0% of sodium bisulfite and 0.5% of sodium carbonate to the extraction water. However, the amount of ashes in the extract, mainly represented by the added salts, was equal to 26.5%.

The separation of the phenolic extracts from the inorganic salts has been performed at the laboratory scale by ultrafiltration or dialysis. The use of such technology at the industrial scale is unlikely, as pointed out by Jorge et al. (1999), and a careful evaluation of the most convenient amount of chemicals to add to the extraction water, in order to avoid disadvantageous dilution of the phenolic compound in the crude extracts, is therefore needed.

The addition of sodium carbonate produced more minor effects than sodium hydroxide or sulfites. Furthermore, self-gelling of Loblolly pine extracts after being stored for 2 days at room temperature was observed (Chen and Pan 1991).

Improvement in the total extraction yield was also observed after addition of urea in the extraction of Radiata pine and Norway spruce bark (Sealy-Fisher and Pizzi 1992; König and Roffael 2003), but also in these cases the gain in total yield could mostly be related to the presence of urea in the final dry extract.

Quite singular results were reported by Vázquez Penas et al. (1992), who performed a three-level factorial experimental design analysis on the extraction of Maritime pine bark, varying the liquid:bark ratio, concentration of sodium hydroxide and temperature of extraction. This resulted in negative effects on the total yields when any of the process variables were increased, in disagreement with most analyses published on the topic.

2.4.4 External factors

Variables not directly related to the extraction process, such as the quality of the collected bark, showed a noteworthy influence on extract characteristics. Weissmann (1984)

performed hot water extractions on Norway spruce bark collected from trees felled at different times from March to October. The average total yield was 250 g/kg_{DB}, and total carbohydrates were approximately 40% w/w in all the extracts. No seasonal trends were detected in total carbohydrates and phenolics, but a regular increase of the mono-/oligosaccharides fraction, in parallel to the corresponding decrease of the extracted polysaccharides, was detected from the late spring to the end of the vegetation period.

Higher phenolic yields were observed from Norway spruce bark collected between 11 m and 35 m trunk height than in samples collected between 0 m and 11 m (König and Roffael, 2002). The different cambial age of the bark samples was attributed to this finding.

The high variability of the results within the same softwood species was observed by Chupin et al. (2013), who performed identical alkaline water extractions on bark samples collected from five distinct Maritime pine logs, resulting in 1.5-fold variations in total yield and 5-fold variations in phenolic yield across the trees.

2.4.5 Modification of condensed tannins during extraction

The extraction of condensed tannins with hot water might induce a reorganization of their structure. The most known and likely modifications are phlobatannin rearrangement, heterocycle opening, interflavanoid link cleavage, and oligomer autocondensation (Ferreira et al. 1992). These mechanisms compete with each other, and the predominance of a particular reaction is a function of both the environment and the tannin type. In alkaline conditions resorcinol tannins (e.g. wattle, quebracho) mostly show phlobaphenes rearrangement (Ferreira et al. 1992; Pizzi and Stephanou 1994a). However, interflavanoid link cleavage followed by quick recombination in a different configuration (e.g. from linear to angular tannin) are characteristics of phloroglucinol tannins (pine, pecan nut) at the same conditions (Pizzi and Stephanou 1994a). All the described rearrangements are also considered likely in an acid environment (Hemingway and McGraw 1983; Bae et al. 1994). The addition of sulfites favors the interflavanol bond cleavage and the heterocycle opening, resulting in sulfite substitutions at positions C4 and C2 that enhance the tannin solubility (Foo et al. 1983). Tannins extracted with the aid of sulfites from *Radiata* pine bark showed a higher fraction of smaller oligomers ($MW_N < 1000$ Da) than those extracted with pure hot water (Yazaki and Hillis 1980). A partial depolymerization of the extracted tannins was indicated.

In phloroglucinol tannins the interflavanoid link cleavage and opening of the heterocycle ring, both naturally occurring or triggered by the addition of sulfites, might result in the autocondensation of tannin oligomers through the development of C4-C8, C4-C6, C2-C8 interflavanol bonds (Bae et al. 1994; Pizzi and Stephanou 1994a). Stable and poorly soluble products (phlobatannins, phlobaphenes) are thus formed during the extraction, both

in situ and in the extraction water, in which they precipitate. The addition of stronger nucleophiles like phloroglucinol, m-phenylen-diammine or urea might limit this effect (Sealy-Fisher and Pizzi 1992). The nucleophile should react more quickly with the cleaved or opened tannins than another oligomer, therefore preventing autocondensation.

The catechin acid rearrangement of procyanidins, which inhibits the tannin's reactivity towards formaldehyde, is less common and limited to the terminal unit for steric hindrances (Pizzi and Stephanou 1994a). The reaction is favored in the presence of phloroglucinol (Ferreira et al. 1992) and during long permanence of the extracts in alkaline conditions at temperatures between 40 and 50°C (Navarrete et al. 2011).

2.4.6 Post treatments of the extracts

The high concentration of carbohydrates in softwood bark extract has been regarded as their major shortcoming. Purification processes with the aim to remove the co-extracted carbohydrates from the extracts have therefore been considered.

Purified Radiata pine and European larch bark extracts were obtained by Weissman (1985b) exploiting the scarcer solubility of pectins in alcohols than phenolic compounds. The dry crude extracts were rinsed with ethanol and the solute collected. Even if only 20% of the total carbohydrates were removed from the extracts, the viscosities of the purified extracts dropped significantly in comparison to the crude samples (Figure 8).

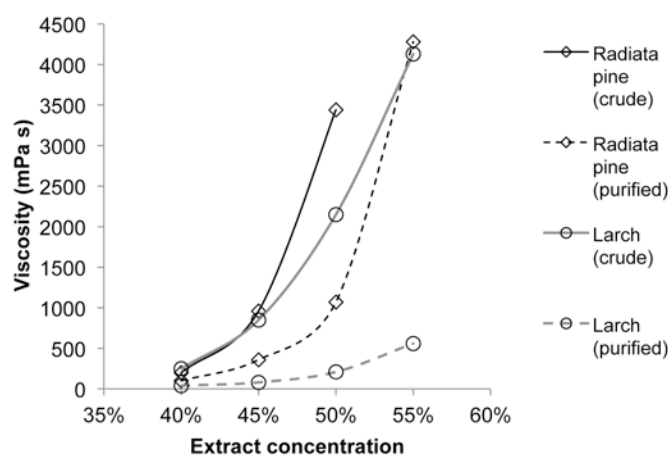


Figure 8: Viscosity of aqueous solutions of bark extract from Radiata pine and European larch at different concentrations. Crude and ethanol purified extracts are shown (based on the data from Weissmann 1985b).

Similar results were obtained by Yazaki and Hillis (1980) after separation of methanol soluble and methanol insoluble compounds from Radiata pine extracts. While the viscosity of the crude extract was equal to 85 mPa·s, that of the methanol soluble fraction, which

represented 87% of the crude extract, resulted in merely 5 mPa·s. A concomitant slight increase in the Stiasny number of the methanol fraction was also observed. The insoluble fraction showed a viscosity higher than the measuring range of the used equipment and a strong decrease in the Stiasny number.

The difference in MW_N between tannins and pectins was exploited by Yazaki and Hillis (1980) to purify Radiata pine extracts by molecular filters. Ultrafiltration through a 10^6 Da membrane gave a filtrate (yield = 77.6% w/w) with a viscosity 10 times lower than crude extracts. A reduction in the Stiasny number of the filtrate suggested that the filter retained a considerable amount of tannins. The separation of tannins from smaller compounds, e.g. phenolic monomers and monosaccharides, was achieved by ultrafiltration with 10^4 Da membranes (Yazaki 1980). The retentate was equal to 62.5% w/w of the crude extracts and showed a higher Stiasny number.

2.4.7 Alternative extraction methods

Hot water extraction of Radiata pine bark at high pressures and temperatures (4-10 bars, 100-180°C) were performed by Inoue et al. (1998). Decreased yields and higher concentrations of non-phenolic compounds in the pure water extracts were observed as both temperature and pressure increased. In alkaline conditions, both the total and phenolic yield increased and became less dependent on temperature and pressure

Barks from various pine species were extracted using supercritical CO_2 (Yesil-Celiktas et al. 2009a). Total phenolic concentrations in extracts were between 2% and 8% w/w, suggesting poor solvability of phenolic compounds in CO_2 at the applied conditions.

Soxleth, microwave and ultrasound assisted extraction, showed better extraction yields than simple maceration (Aspé and Fernandez 2011). Microscopy of bark samples extracted with this method showed a severe disruption of the cell structure. Macerated and stirred samples appeared much less damaged.

2.4.8 Industrial extraction

The industrial extraction of tannins is generally performed in hot water through a multi-stage counter-current process. A schematic description of an industrial extraction plant was reported by Li and Maplesden (1998) and used as a basis to sketch the process flowchart in Figure 9.

Air-dried raw bark is hammer-milled and screened to between 1 and 4 mm in size. Any smaller might result in a problematic handling of the raw material, and any larger would not be suitable for efficient diffusion of the extractives through the tissue. The bark is loaded into an extraction tank and percolated with the water already used in the previous extraction

steps. The ratio between water and bark should be higher than 5:1, as the very porous softwood bark can absorb water up to four times its own dry weight. The extraction time of each step is usually between 30 and 60 min. After the first extraction step, the bark is again extracted with water that has already percolated through all the extraction steps except the last one. Four to eight successive extraction steps in separate tanks are generally performed. In the last step the bark is extracted with clean hot water at 70-90°C. The process permits the operation of as many parallel extractions as the number of extraction stages. Generally no re-heating of the water is performed between the tanks. A press filter after the discharge of the bark from the last extraction tank might be utilized to further recover the remaining water absorbed in the bark.

At the end of the extraction process, the extraction water has a dry content between 1% and 5%, depending on the extraction yield. After filtration, it is concentrated in an agitated evaporator to 30-40% in dry content. Eventually, the concentrated extract is dried to a fine powder by spray-drying.

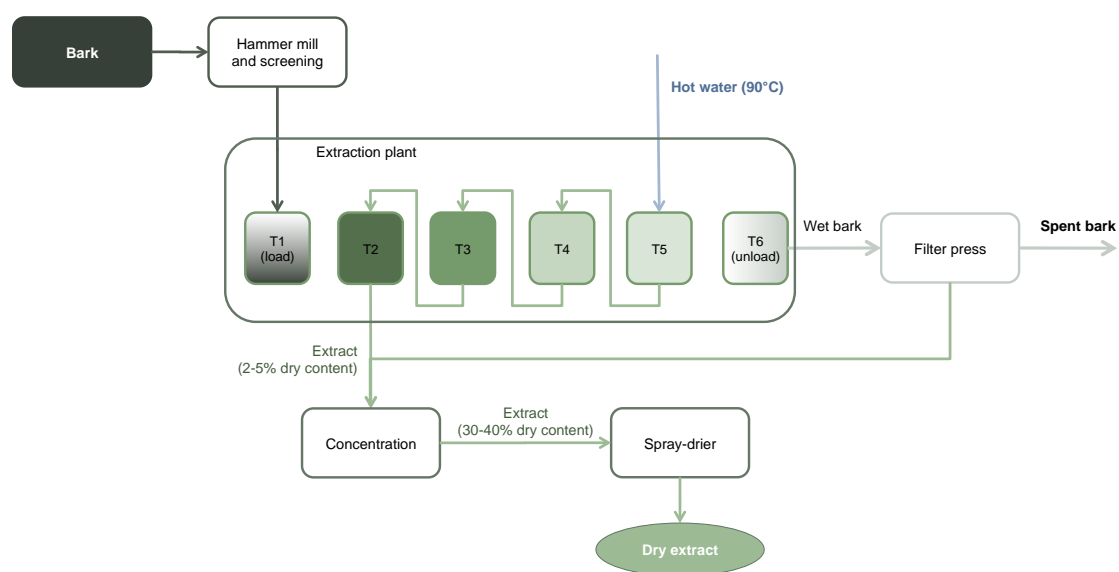


Figure 9: Flowchart of a tannin extraction plant with 4 successive extraction stages.

3. Experimental

The methods used in the bark sampling, extraction and analysis were described in detail in the publications related to this thesis (Bianchi et al. 2014, 2015, 2016). In the following sections, only a brief description of these experimental procedures is reported. The methods concerning additional investigations that were not reported in the appended publications are instead fully described.

3.1 Bark sampling

Bark flakes from Silver fir (*Abies alba* [Mill.]), European larch (*Larix decidua* [Mill.]), Norway spruce (*Picea abies* [Karst.]), Douglas fir (*Pseudotsuga menziesii* [Mirb.]) and Scots pine (*Pinus sylvestris* [L.]) were collected in the early spring from harvested logs in a forest in the Swiss Jura close to Biel (Figure 10). The logs were felled 1 to 3 months before bark collection and had a breast height diameter between 40 and 60 cm (Bianchi et al., 2015).



Figure 10: Sampling of Norway spruce bark flakes using a debarking iron

For the analysis of the influence of bark outdoor storage on the extracts' characteristics, a Norway spruce log with a breast height diameter of 50 cm was felled at the end of October in the same forest, brought the day after in a log-yard in Biel and left exposed to natural direct weathering for 15 months. Bark flakes at different positions along

the log length were regularly collected from the day of harvesting up to the end of storage (Bianchi et al., 2016).

All the collected samples included both inner and outer bark. The bark flakes were deep-frozen and stored at -20 °C directly after collection and protected from light and air until further analysis or extraction.

3.2 Bark topochemistry and composition

The anatomical structure of bark was investigated by scanning electron microscopy (SEM) of freeze-dried bark chips previously coated with gold-palladium (Bianchi et al. 2016). The localization of the phenolic deposits within the bark tissue (topochemistry) was analyzed by UV-microspectrometry (UMSP) (Koch and Kleist, 2001; Bianchi et al. 2016). More in detail, semi-thin transverse sections of bark were scanned with a geometric resolution equal to 0.25 μm x 0.25 μm and the UV-absorbance at 280 nm was recorded. Point measurements were performed at different anatomical sites on a spot of 1 μm^2 in size and the UV-visible absorbance spectrum between 240 and 500 nm was recorded.

The amount and monomeric composition of the bark carbohydrates were analyzed by two-step hydrolysis, followed by high performance anion exchange chromatography with Borat post-derivatization (Borat-HPAEC), as reported by Willför et al. (2009).

The nature and total amount of extractives in the barks were analyzed by successive extractions at increased solvent polarities as described in Table 2. The extractions were performed on an accelerated solvent extraction device (ASE 200 Dionex®) equipped with 22 mL cells. About 2 g of freeze-dried bark milled with a vibratory disc mill (average particle size \approx 50 μm) were loaded in the cell. All extractions were repeated 3 times. Petrol ether extracts were dried by evaporation at room temperature. Acetone extracts were vacuum-dried at 40°C using a Büchi Syncore® device. Water extracts were freeze-dried. Eventually, the weights of the different dry extracts were recorded.

Table 2: Scheme of the process used for the characterization of the total extractives in softwood barks.

Extraction step	Solvent	Temperature (°C)	Heating time (min)	Extraction time (min)	Extraction cycles
A	Petrol ether	70	5	10	2
B	Acetone:Water 90:10 v/v	100	5	10	2
C	Water	120	6	10	2

Total procyanidins and prodelphinidins in bark were estimated by direct thiolysis on milled barks using cysteamine hydrochloride in acidic methanol (Bianchi et al. 2016). The quantification of the cleaved flavanol monomeric units was performed by reverse phase high

performance liquid chromatography with UV detection at 280 nm (HPLC-UV). Calibration was performed using (-)-epicatechin, (+)-catechin, (-)-gallocatechin and (-)-epigallocatechin analytical standards (Sigma Aldrich, Switzerland).

3.3 Bark extractions

Extractions of softwood bark were performed using an ASE 200 Dionex® device (Bianchi et al., 2015). Unless reported otherwise, all the extractions were performed on 2 g of bark in water at 60°C, 100 bar and lasted 15 min (including cell heating). In some extractions, sodium sulfite and/or urea were added to the extraction water in concentrations equal to 2.5 or 5 g/L (Bianchi et al., 2016). Prior to extraction the barks were freeze-dried and ground to fine powder ($\approx 50 \mu\text{m}$) with a vibratory disc mill. To analyze the influence of the bark particle size on the extraction yield, Norway spruce bark was also ground with a hammer-mill (mesh = 2 mm) and chopped manually to chips of about 10 mm. All extractions were repeated at least 3 times. The extracts were eventually freeze-dried (Figure 11) and stored protected from light and air.



Figure 11: Freeze dried hot-water extracts from the bark of a) Silver fir, b) European Larch, c) Norway spruce, d) Douglas fir, e) Scots pine.

3.4 Extract composition analysis

The hot water bark extracts were characterized according to phenolic monomers, phenolic oligomers, mono- and oligosaccharides, polysaccharides and inorganic compounds (Bianchi et al., 2015). A flow chart of the analytical scheme applied is reported in Figure 12.

Briefly, the crude extracts were separated by solid phase extraction (SPE) into three fractions (F0, F1 and F2) following the procedure described by Sun et al. (1998) with slight

modifications. The fractions were associated to mono-/oligosaccharides and phenolic acids (F0), phenolic monomers and smaller oligomers (F1), and larger phenolic oligomers (F2). Total phenolic compounds in the crude extract and the three fractions were estimated as (-)-epicatechin equivalents (ECEg) using the Folin-Ciocalteu assay (Singelton et al., 1999). Total phenolic compounds in fraction F2 were also estimated as quebracho tannin equivalents (QEG), using highly purified quebracho heartwood extracts for the calibration (Fintan QS – Silvateam S.p.A., Italy).

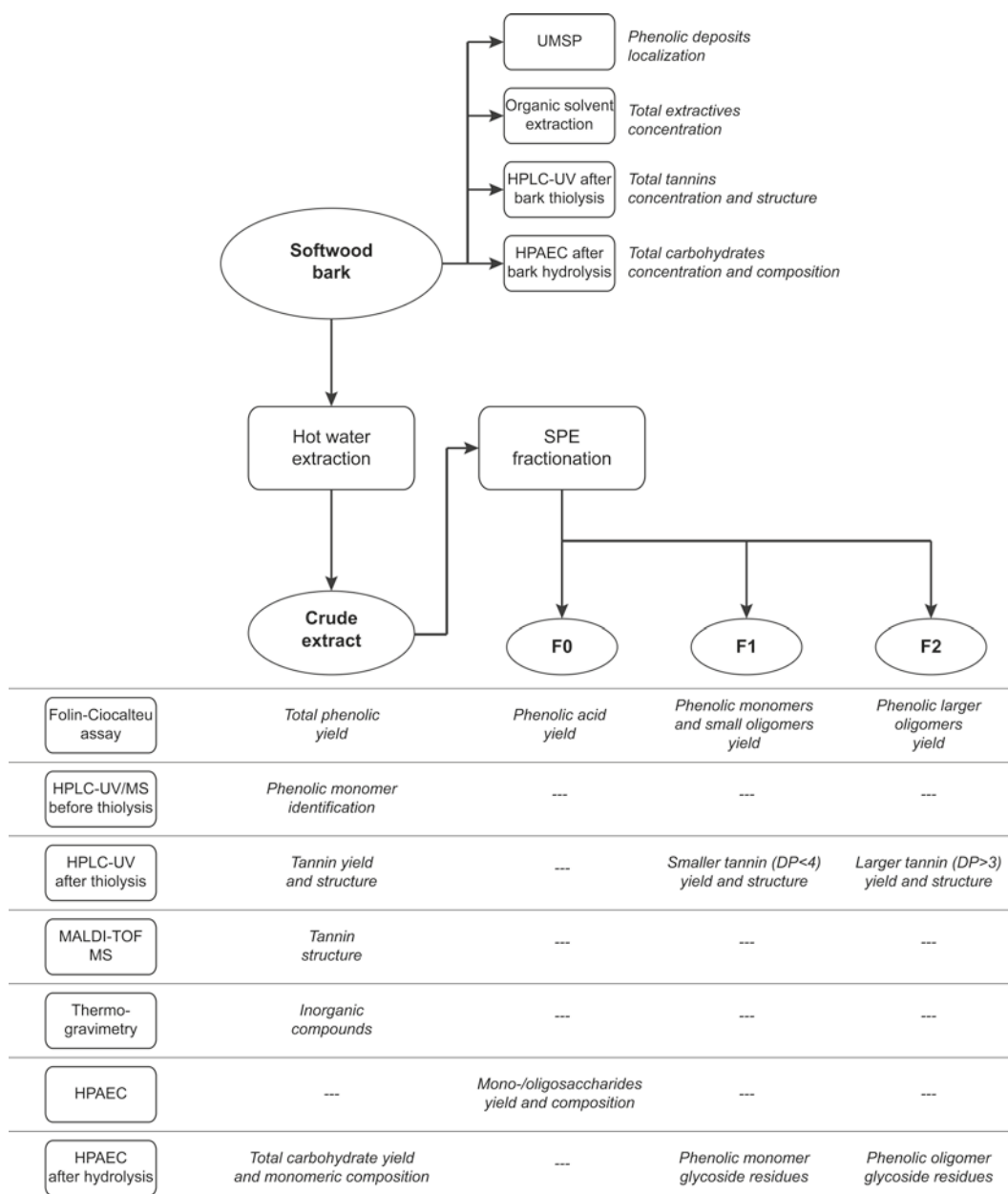


Figure 12: Scheme of the analytical procedure applied for the analysis of the bark and bark extracts (DP = degree of polymerization)

The total amount and monomeric composition of the extracted carbohydrates were determined by acid hydrolysis of the crude extracts followed by Borat-HPAEC and HPAEC with pulsed amperometric detection (PAD) (Willför et al., 2009; Manns et al., 2014; **Bianchi et al., 2015**). The presence and identification of mono- and oligosaccharides in the extracts (free carbohydrates) were analyzed with Borat-HPAEC and HPAEC-PAD on the fraction F0, without previous hydrolysis. The amount and composition of bound carbohydrates, which include both polysaccharides and glycosyl residues, were evaluated as the difference between total and free carbohydrates. The presence of phenolic glycosides in F1 and F2 was examined by Borat-HPAEC before and after acid hydrolysis of the fractions.

The amount of inorganic compound was determined as ashes after thermogravimetry of the crude extracts up to 650°C (**Bianchi et al., 2015**).

The identification of some of the phenolic monomers present in the crude extracts was performed through the analysis of HPLC-UV/MS mass spectra and MALDI-TOF mass spectra in the range between 300 and 900 m/z (**Bianchi et al., 2015**).

3.5 Condensed tannin characterization

The identification of the flavan-3-ol monomeric composition of the extracted condensed tannins was performed by matrix assisted laser desorption ionization - time of flight mass spectrometry (MALDI-TOF MS) (**Bianchi et al., 2014**) and acid thiolysis followed by reverse phase HPLC-UV (**Bianchi et al., 2015**).

MALDI-TOF MS of crude extract samples were prepared in aqueous acetone solutions spiked with potassium salts to avoid any interference from already dissolved cations (e.g. Ca⁺, K⁺, Mg⁺) in the extracts. 2,5-dihydroxybenzoic acid was used as matrix. Mass spectra were collected in the range between 900 and 5000 m/z.

Thiolysis was performed in acid methanol with cysteamine hydrochloride on crude extracts and fractions F1 and F2. HPLC elution gradient of the mobile phase (acidic water and acidic acetonitrile:water 4:1 v/v) was finely tuned to achieve good separation of the peaks corresponding to flavan-3-ols and their thioethers. Detection was performed with a UV-diode array at 280 nm. The quantification of the cleaved flavan-3-ol and their thioether was performed by calibration with (-)-epicatechin, (+)-catechin, (-)-gallocatechin and (-)-epigallocatechin analytical standards (Sigma Aldrich, Switzerland). The mean degree of polymerization (mDP) of condensed tannins was assessed by the molar ratio between total cleaved units and the cleaved terminal units (released as native flavan-3-ols), following the procedure described in Jerez et al. (2007a). The determined mDP can be equated to the number-average polymerization degree (X_N) of SEC measurements.

4. Results and discussion

4.1 Softwood bark anatomy and chemistry

4.1.1 Softwood bark anatomy

Scanning electron microscopy (SEM) pictures of bark samples from Silver fir, European larch, Norway spruce, Douglas fir and Scots pine displayed similar anatomical structures (Figure 13). Parenchyma cells represented the large majority of inner and outer bark. Within outer bark, successive periderm bands were clearly visible, specifying the growth development of the bark tissues. The periderm bands were generally formed by 4 to 5 layers of thick walled sclereids tissue (or “sclerotic phelloid”). One to two layers of thin walled cork cells were observed outerwards the sclerotic phelloids. Exceptions to this general feature were European larch, which showed more than 10 sclerotic phelloid layers in each periderm, and Douglas fir, which had periderms almost completely represented by collapsed cork cells and few sclereids. Isolated sclereids, or clusters of them, were observed within parenchyma cells, and particularly in Silver fir, Douglas fir and European larch.

Solid deposits in the majority of the sclereids and cork cells were observed (Figure 13). Most of the deposits almost completely filled the lumen, while others simply coated the innermost cell walls (S3). Among parenchyma cells, deposits occurred far less frequently. The scattered presence of bright squared granules was observed in all species and associated to calcium oxalate salts (Paremeswaran et al. 1976).

A thorough analysis of the Norway spruce bark anatomy with additional SEM pictures is reported in **Bianchi et al. (2016)**.

4.1.2 Softwood bark composition

Total extractives and carbohydrates in softwood bark tissues were determined by successive extractions at increasing solvent polarity (petrol ether, acetone:water 90:10 v/v, water) and two-step acid hydrolysis followed by Borat-HPAEC, respectively. The estimations of the total amount of tannins (as procyanidins and prodelphinidins) within the bark tissues and of their mean degree of polymerization (mDP) were performed by direct thiolysis on finely milled bark.

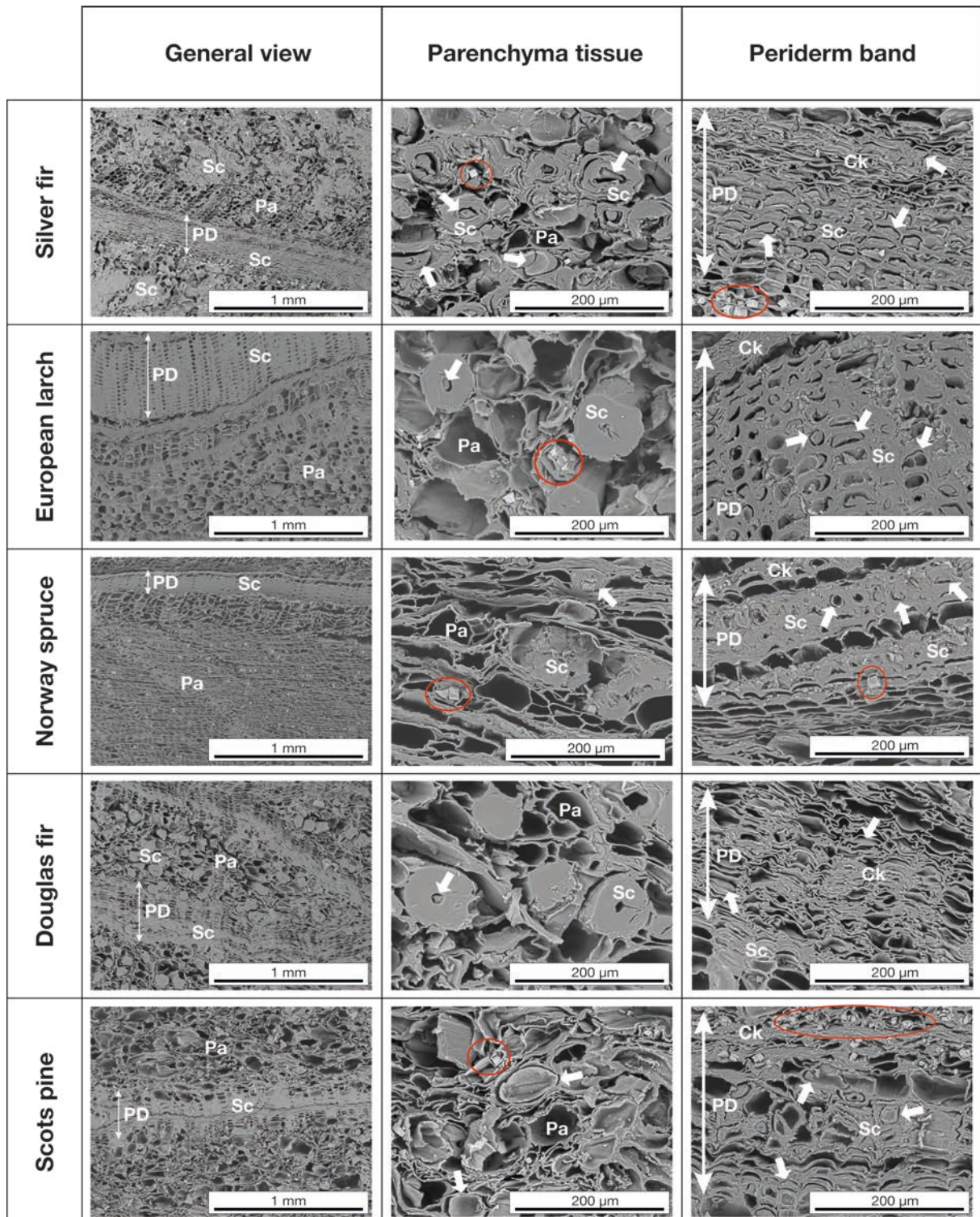


Figure 13: SEM pictures of the bark of different softwood species. Periderm bands (PD), parenchyma cells (Pa), sclereids (Sc), cork cells (Ck), solid deposits (white arrow) and calcium oxalate crystals (red circle) are evidenced.

Total extractives represented between 112 and 251 g/kg of the dry bark (g/kg_{DB}) (Table 3). Scots pine and European Larch corresponded to the lowest and the highest yields, respectively. These values were slightly lower than those reported in other studies for the same species (Table 1), especially for Scots pine and Norway spruce. Dissimilarities in the extraction protocols were considered as a rationale for the observed differences. The relative trend across the species is however consistent with the literature data and, in particular, the considerably lower amount of total extractives showed by Scots pine bark compared to other species.

Table 3: Total extractives and condensed tannin in softwood bark (g/kg of dry bark)
Extractives obtained by successive extractions with solvents at increasing polarities
Tannin amount and mean degree of polymerization (mDP) estimated by direct thiolysis on milled bark followed by HPLC-UV (mean ± std.dev., n=3)

	Silver fir	European larch	Norway spruce	Douglas fir	Scots pine
EXTRACTIVES					
Petrol ether	18 ± 2	20 ± 2	21 ± 1	21 ± 1	21 ± 3
Acetone:Water	120 ± 4	183 ± 14	87 ± 1	167 ± 5	49 ± 5
Water	52 ± 1	49 ± 10	65 ± 4	49 ± 3	42 ± 2
Total	190 ± 3	251 ± 13	173 ± 3	237 ± 4	112 ± 4
TANNINS					
Procyanidins	9 ± 1	56 ± 6	27 ± 3	25 ± 3	16 ± 2
Prodelphinidins	39 ± 4	n.d.	n.d.	n.d.	n.d.
Total	48 ± 5	56 ± 6	27 ± 3	25 ± 3	16 ± 2
mDP	3.5 ± 0.4	5.0 ± 0.5	7.0 ± 0.7	4.1 ± 0.4	7.8 ± 0.8

n.d. = non detected

Non-polar compounds, extracted with petroleum ether at 70°C, were between 18 and 21 g/kg_{DB} (Table 3). They were largely associated with fatty and resin acids (Fengel and Wegener 1983b; Valentin et al., 2010; Krogell et al., 2012).

Polar compounds were extracted with both acetone and hot water, and represented phenolic monomers, oligomers, monosaccharides and pectins (Timell, 1961; Dietrich et al., 1978; Weissman, 1981; Krogell et al., 2012). The biggest variations among the different softwood species were detected in the acetone extracts (49 – 183 g/kg_{DB}; Table 3), which should be predominantly associated with phenolic monomers and oligomers. Hot water extracts, mainly correlated to the larger phenolic oligomers and pectins, had more homogeneous yields (42 – 65 g/kg_{DB}; Table 3). For Silver fir, European larch and Douglas fir the acetone extracts clearly prevailed, while in Norway spruce and Scots pine their amounts were comparable to the water extracts. A higher concentration of large phenolic oligomers in the phenolic extractives of Norway spruce and Scots pine was therefore indicated.

The total amount of tannins, as sum of the cleaved procyanidin and prodelphinidin units after bark thiolysis, ranged from 16 to 60 g/kg_{DB} (Table 3). These values were much

lower than the sum of acetone and water extraction yields, suggesting that procyanidins and prodelphinidins represented a small fraction of the phenolic extractives. The measured concentrations of total tannins could however be severely underestimated if oligomers other than pure linear procyanidins or prodelphinidins were present. I.e. fisetinidins and robinetinidins cannot be cleaved by thiolysis, branched tannins are less efficiently depolymerized than linear oligomers, and the substitution of flavanol units with different monomers might result in thiolysis-resistant bonds (Hemingway and McGraw 1983; Steynberg et al. 1994; Matthews et al. 1997b).

Tannins were attributed to procyanidins in all softwood species except Silver fir, for which prodelphinidins partially substituted by procyanidins units were detected. This finding is quite unusual. Prodelphinidins have been commonly found in softwood cones and needles, but only few pine or fir species showed their occurrence in bark (Hemingway 1989; Porter 1989). Silver fir bark showed the shortest oligomers (mDP = 3.5), while Norway spruce and Scots pine presented the longest (mDP = 7.0 and 7.8, respectively) (Table 3).

The total amount of bark carbohydrates considerably varied across the species (Table 4). The lowest concentrations were detected in Douglas fir (25.1%) and European larch (26.2%), while the highest corresponded to Silver fir (41.8%). Norway spruce and Scots pine showed intermediate values (36.7% and 32.7%, respectively). Cellulose, correlated to the glucose residues after acid hydrolysis of the bark, varied between 14.5% and 28.2%, in agreement with previous determinations (Timell 1961; Dietrich 1978; Fradinho et al. 2002; Valentin et al. 2010; Krogell et al. 2012). Despite the noticeable variations in the absolute values among the softwood species, cellulose corresponded to approximately 60% of the total carbohydrates in all barks.

The monomeric composition of carbohydrates (Table 4) indicated the presence of typical softwood bark polysaccharides such as galactoglucomannans, arabino-4-*O*-methylglucuronoxylans, galactans, arabinogalactan and arabinans (Timell, 1961; Fu and Timell, 1972a; Dietrich et al. 1978; Krogell et al., 2012; Le Normand et al., 2014). The variations in the relative ratios among the monomeric residues suggested changes in the bark polysaccharide composition among the softwood species, e.g. mannans were more prevalent in Silver fir and European larch, galactans more typical for Norway spruce and Scots pine, and arabinans less frequent in European larch.

The polysaccharide compositions detected in the bark of softwood species substantially differed from those for the corresponding xylem. A larger prevalence than in bark of mannose and xylose residues, associated to galactoglucomannans and arabino-4-*O*-methylglucuronoxylans, are in fact generally reported for softwood xylems, while pectic polysaccharides such as galactans, arabinogalactan and arabinans have been detected in minor amounts (Fengel and Wegener 1983a). A peculiar case is represented by European larch bark, which showed a modest amount of galactose monomer residues (1.6%) in

contrast with the known and characteristic high concentration of arabinogalactans (10-25%) of larch xylem (Fengel and Wegener 1983a).

Table 4: Carbohydrate monomeric composition in softwood bark (% w/w on dry bark)
Monomeric carbohydrate residues determined by two-step acid hydrolysis of milled bark followed by Borat-HPAEC (mean \pm std.dev., n=3).

	Silver fir	European larch	Norway spruce	Douglas fir	Scots pine
Glc	28.2% \pm 0.5%	16.1% \pm 1.2%	21.5% \pm 0.7%	14.5% \pm 0.5%	20.1% \pm 0.5%
Xyl	3.4% \pm 0.1%	2.4% \pm 0.1%	4.3% \pm 0.1%	2.5% \pm 0.1%	3.2% \pm 0.1%
Gal	1.7% \pm 0.1%	1.6% \pm 0.1%	4.1% \pm 0.1%	1.9% \pm 0.1%	3.6% \pm 0.1%
Ara	2.5% \pm 0.1%	1.3% \pm 0.1%	3.3% \pm 0.2%	2.5% \pm 0.1%	2.9% \pm 0.1%
Man	5.6% \pm 0.1%	4.4% \pm 0.1%	2.9% \pm 0.1%	3.3% \pm 0.1%	2.5% \pm 0.1%
Rha	0.4% \pm 0.1%	0.3% \pm 0.1%	0.5% \pm 0.1%	0.3% \pm 0.1%	0.5% \pm 0.1%
Total	41.8% \pm 0.8%	26.2% \pm 1.0%	36.7% \pm 0.5%	25.1% \pm 0.5%	32.7% \pm 0.7%

4.1.3 Topochemistry of Norway spruce bark

The distribution and the nature of the phenolic compounds in spruce bark were investigated by mapping the UV absorbance at 280 nm (A_{280}) on transversal sections (Bianchi et al. 2016). The deposits in the lumen of sclereids and cork cells are clearly silhouetted against the surrounding bark tissues with their distinctly higher A_{280} (Figure 14). Their phenolic nature was then evidenced. In agreement with the SEM observations, deposits in the sclereids occurred as a bulky material in the cell lumen (Sdb) or as a layer coating the S3 cell wall (SDc) (Figure 14). In cork cells only bulky deposits were observed (CD) (Figure 14).

Point measurements of the bark cell deposits (Figure 15) showed a broad peak with a maximum at around 285 nm and significant absorbance up to 400 nm. This type of spectrum is characteristic of conjugated double bonds or carboxylic groups (Goldschmid, 1975). The presence of highly condensed phenolic compounds in the deposits was therefore suggested. These substances could be attributed to condensed tannins, but the presence of more oxidized structures such as phlobaphenes was not excluded. The deposits in the outer sclereid (Sdb outer) and cork cell (CD) showed a broader absorbance region than those in the inner sclereids (Sdb inner) (Figure 15). An increase in the degree of condensation of the phenolic deposits towards the most external periderm cells was indicated. Differences in the absorbance spectra between the bulky and layer deposits (Sdb and SDc) were also observed (Figure 15). Different classes or compositions of phenolic compounds were therefore expected in the two kinds of deposits.

The S2 cell walls of Norway spruce bark were indicated by an A_{280} between 0.32 and 0.78 (Figure 14). This range is appreciably higher than the A_{280} in Norway spruce xylem cell

walls (0.10 – 0.41; Koch and Kleist 2001). The absorbance spectra of the walls of parenchyma cells (PW) and sclereids (SW) (Figure 15) showed a maximum around 280 nm and a minimum at about 260 nm, typical of guaiacyl lignin (Musha and Goring, 1975). A high lignification of these cell walls was thus indicated. Differently, cork cell walls (CW) showed a broader absorbance spectrum (Figure 15), suggesting the presence of phenolic compounds different from lignin incrustated in this tissue, as already described for beech bark by Prislán et al. (2012).

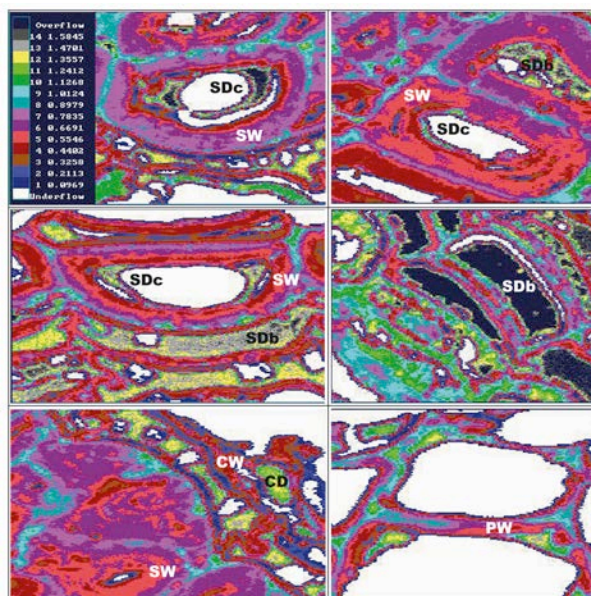


Figure 14: UV-absorbance maps (280 nm) of Norway spruce bark transverse sections. Representative bark spots are highlighted: SDb = bulky deposits in sclereid lumen; SDc = layer-shaped deposits coating the inner S3 sclereid wall; CD = bulky deposits in cork cells; SW = S2 cell wall of sclereids; CW = S2 cell wall of cork cells, PW = S2 cell wall of parenchyma cells (modified from Bianchi et al. 2016).

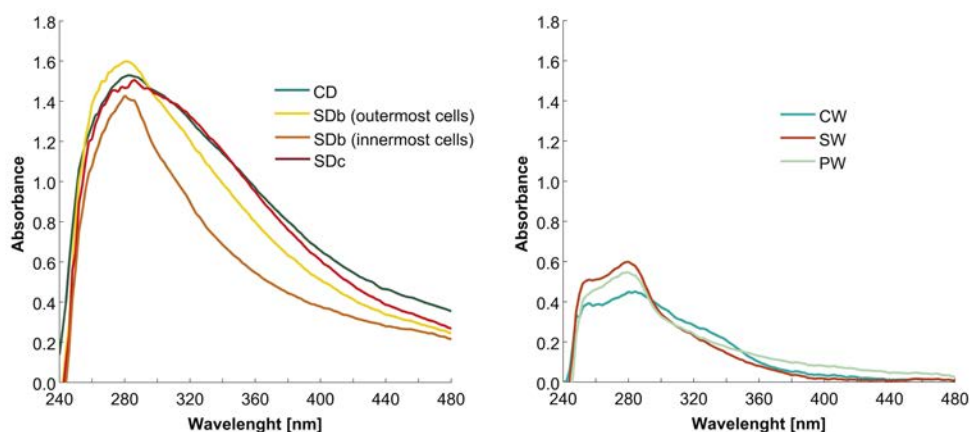


Figure 15: Point UV-absorbance spectra (240-500 nm) of different anatomical spots in Norway spruce bark (see Figure 14). CD = cork cell lumen deposits, SDb = bulky sclereid lumen deposits, SDc = thin layer sclereid lumen deposits, CW = cork cell S2 walls, SW = sclereid S2 walls, PW = parenchyma S2 walls (modified from Bianchi et al. 2016).

4.2 Softwood bark hot water extract characterization

4.2.1 Total yield and extract composition

Hot water extracts of the analyzed softwood bark showed considerable differences in both yield and composition among tree species (Table 5) (Bianchi et al., 2015). Total yields of extraction performed at 60°C were between 26.9 and 120.2 g/kg_{DB} (Table 5), the lowest value associated to Scots pine and the highest to Silver fir. These values are in agreement with those measured in other studies on European softwood species (Weissmann 1981, 1985; Bertaud et al. 2012; Kemppainen et al. 2014), but considerably lower than the yields reported for the currently used tannin-rich extracts of Black wattle bark and quebracho heartwood, which are between 150 and 425 g/kg_{DB} (Sealy-Fisher and Pizzi 1992; Roffael et al. 2000; Hoong et al. 2011). The total yields of hot water extraction for European larch, Norway spruce and Douglas fir (Table 5) was about one third of the total amount of bark extractives determined by successive extractions with petrol ether, acetone and water (Table 3). In Scots pine the efficacy of water extraction was reduced to one fourth of the total extractives. In contrast, two thirds of the total extractives were recovered by Silver fir water extraction. A limited efficiency of hot water extraction at 60°C was therefore indicated.

Table 5: Total yield of softwood bark extracts and their phenolic, carbohydrate and inorganic (ashes) compositions. Phenolic compounds expressed as (-)-epicatechin equivalents (ECEg). (mean ± std.dev., *n* samples)

	Total yield (<i>n</i> = 5) g/kg _{DB}	Phenolics (<i>n</i> = 3) ECEg/g dry extract	Carbohydrates (<i>n</i> = 1) g/g dry extract	Inorganics (<i>n</i> = 3) g/g dry extract
Silver fir	120.2 ± 2.4	27.9% ± 0.4%	29.1% ± 0.3%*	3.8% ± 0.4%
European larch	91.7 ± 4.5	45.6% ± 0.2%	20.9% ± 0.3%*	4.6% ± 0.5%
Norway spruce	54.5 ± 4.7	34.2% ± 0.1%	30.3% ± 0.3%*	3.1% ± 0.3%
Douglas fir	75.7 ± 2.1	30.8% ± 0.2%	41.5% ± 0.3%*	1.5% ± 0.2%
Scots pine	26.9 ± 1.9	13.0% ± 0.1%	45.7% ± 0.3%*	5.2% ± 0.5%

* estimated analytical error based on previous measurements
ECEg = g of (-)-epicatechin equivalents
kg_{DB} = kg of dry bark

All softwood bark extracts were mainly represented by both phenolic compounds and carbohydrates (Table 5). Inorganic compounds represented a minor component of the extracts, between 1.5 and 5.2%, (Table 5). They were associated with inorganic compounds like potassium, magnesium and phosphorous ions, the most common constituents of soluble salts in barks (Saarela 2005; Rothpfeffer and Karlton 2007; Krogell et al. 2012).

The relative ratio between phenolics and carbohydrates varied between species. European larch showed the highest concentration of phenolics (45.6%) and the lowest in

carbohydrates (20.9%), while Scots pine showed the opposite, i.e. 13.0% of phenolics and 45.7% of carbohydrates.

The total concentration of phenolic was determined as (-)-epicatechin equivalents (ECEg) using the Folin-Ciocalteu colorimetric assay. The method cannot allow for different mass absorptivities among phenolic compounds (Singelton et al. 1999; Everette et al. 2010). In particular, phenolic oligomers like the condensed tannins generally present lower absorptivities than the sum of their monomers (Kraus et al. 2003; Everette et al. 2010). Consequently, their actual amount might be underestimated when epicatechin is used as a calibration standard. E.g., Folin-Ciocalteu measurements performed on 1 g/L solution of highly purified quebracho tannins, showed an absorbance corresponding to 0.72 ECEg/L. The total phenolic concentrations in the softwood extracts reported in Table 5 should thus be considered as low boundary estimations of their actual values.

In comparison with the acetone bark extraction yields, which can be roughly attributed to the totality of bark phenolic extractives and ranged between 49 and 153 g/kg_{DB} (Table 3), phenolic extraction yields of hot water extractions were significantly lower (3.5 - 42.8 ECEg/kg_{DB} - Table 6). Even when taking the underestimation of the actual phenolic yields achieved by the Folin-Ciocalteu test into consideration (as discussed above) and recalling that acetone yields were instead gravimetrically determined, the huge differences between the yields suggest that a considerable amount of phenolic extractives was not recovered by the hot water extractions, and remained still available in the extracted bark. The same situation was observed in the comparison between the tannin yields determined by thiolysis on the crude hot water extracts (Table 6) and the total tannin amounts determined by direct thiolysis on milled bark (Table 3). Extracted tannins merely represented between 11% (Scots pine) and 33% (Silver fir) of the total tannins. The results indicated that the large majority of bark tannins should be considered as non-extractable with hot water.

A quantification of the ratio between phenolic monomers and oligomers in the extracts was performed through separation of the crude hot water extracts in three fractions by solid phase extraction (SPE) (Bianchi et al. 2015). The fractions, according to Sun et al. (1998) corresponded to:

F0 = carbohydrates and phenolic acids

F1 = phenolic monomers and small oligomers (e.g. dimers, trimers)

F2 = phenolic oligomers

The effectiveness of the SPE fractionation was checked by HPLC-UV. The occurrence of phenolic monomers in the crude softwood bark extracts was evidenced by the presence of UV-absorption peaks in the HPLC chromatograms (Figure 16). After fractionation, the same peaks were detected in the fraction F1. A few small peaks, generally located at lower retention times, were observed in the fraction F0 and ascribed to the most polar phenolic

monomers, such as the phenolic acids. In the fraction F2 no defined peaks were visible, instead only a very broad absorption region at the end of the chromatograms, which is typical of a mixture of different oligomers. A similar but less intense broad absorption region was visible in the chromatograms of the fraction F1, suggesting also the presence of phenolic oligomers in this fraction.

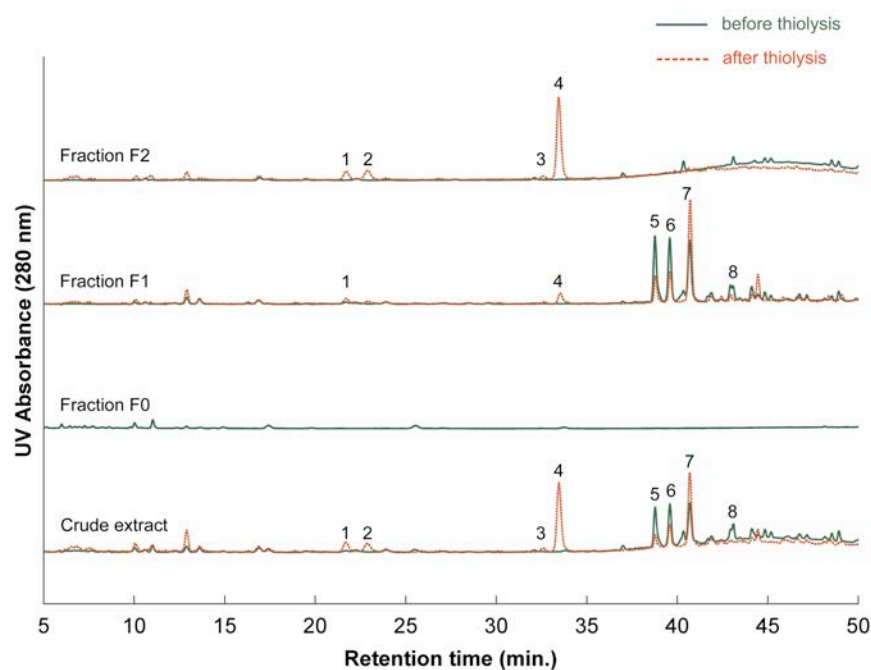


Figure 16: HPLC-UV chromatographs ($\lambda_{\text{abs}} = 280 \text{ nm}$) before and after thiolysis of Norway spruce crude extract and its fractions F0, F1 and F2. The numbered peaks are associated to: 1 = catechin; 2 = catechin thioether; 3 = epicatechin; 4 = epicatechin thioether; 5 = astringin; 6, 7 = taxifolin isomers; 8 = isorhapontin (modified from Bianchi et al. 2016).

The presence of procyanidins and prodelphinidins in both F1 and F2 was confirmed by thiolysis (Figure 16). In Scots pine, Norway spruce and Douglas fir the amount of tannins eluted in fraction F1 was significantly lower than those observed in the corresponding fraction F2. In contrast, European larch and Silver fir showed almost equal amounts of tannins in the two fractions (Table 6). The mDP of the tannins eluted in F1 was between 2.7 and 4.1, while in F2 the range stretched between 3.6 and 8.4 (Table 8). The SPE fractionation method therefore permitted only a partial separation of the phenolic oligomers from the monomers. Practically, fraction F2 was considered as representative of the longest phenolic oligomers, while fraction F1 of a mixture of phenolic monomers and smallest oligomers.

Folin-Ciocalteu assays on the three fractions indicated that phenolic monomers and oligomers (F1 + F2) represented the majority of the extracted phenolics, while phenolic acids occurred in much lower concentrations (Table 6). Fractions F1 and F2, if both estimated as ECEG, showed comparable weights in the extracts. Considering that F2 represented the larger phenolic oligomers, a better estimation of the relative ratio between monomers and

oligomers could be performed expressing F1 as ECEg, and F2 as purified quebracho tannin equivalents (QEg) (Table 6). In accordance to this evaluation, phenolic oligomers represented the majority of the extracted phenolics in all softwood bark extracts except Douglas fir, for which a comparable weight between F1 and F2 was observed (Table 6). For Norway spruce and Scots pine the weight of F2 was almost double that of F1. Even if phenolic monomers likely occurred at lesser concentrations than phenolic oligomers, they represent a considerable part of the extracts, particularly for Douglas fir. Besides the dilution of the tannin concentration in the extracts, the interactions between phenolic monomers and oligomers have not yet been clarified and should be thoroughly investigated.

Table 6: Phenolics' and tannins' yields from hot water extractions of softwood barks

Fractions were obtained by SPE and corresponded approximately to F0 = phenolic acids, F1 = phenolic monomers, F2 = phenolic oligomers. Tannins were estimated by HPLC-UV as the sum of the cleaved flavan-3-ols and relative thioethers after acid thiolysis. (mean \pm std.dev., $n = 3$)

	Crude extract		Fraction F0		Fraction F1		Fraction F2		
	Phenolics ECEg/kg _{DB}	Tannins g/ kg _{DB}	Phenolics ECEg/kg _{DB}	Tannins g/ kg _{DB}	Phenolics ECEg/kg _{DB}	Tannins g/kg _{DB}	Phenolics ECEg/kg _{DB}	QEg/kg _{DB}	Tannins g/kg _{DB}
Silver fir	33.5 \pm 0.5	15.6 \pm 1.3	2.0 \pm 0.1	n.d.	14.0 \pm 0.1	6.7 \pm 0.5	17.4 \pm 0.1	24.2 \pm 0.1	9.0 \pm 0.7
European larch	42.8 \pm 0.2	11.1 \pm 0.8	3.6 \pm 0.1	n.d.	18.4 \pm 0.1	4.0 \pm 0.3	18.6 \pm 0.1	25.6 \pm 0.2	6.2 \pm 0.4
Norway spruce	18.6 \pm 0.1	3.6 \pm 0.4	2.2 \pm 0.1	n.d.	5.8 \pm 0.1	0.5 \pm 0.1	9.9 \pm 0.1	13.8 \pm 0.1	2.6 \pm 0.3
Douglas fir	23.3 \pm 0.1	4.6 \pm 0.5	0.4 \pm 0.1	n.d.	13.1 \pm 0.1	0.8 \pm 0.1	9.1 \pm 0.1	12.6 \pm 0.1	3.5 \pm 0.1
Scots pine	3.5 \pm 0.1	1.7 \pm 0.2	0.1 \pm 0.2	n.d.	1.3 \pm 0.1	0.2 \pm 0.1	1.9 \pm 0.1	2.6 \pm 0.1	1.4 \pm 0.1

ECEg = g of (-)-epicatechin equivalents

QEg = g of purified quebracho tannin equivalents

kg_{DB} = kg of dry bark

n.d. = not detected

Thiolysis on fractions F2 showed a concentration of tannins much lower than the total amount of phenolics expressed as QEg, and especially for Norway spruce, European larch and Douglas fir (Table 6). More extreme thiolysis conditions (e.g. higher temperatures, longer times, higher acidity) didn't significantly improve the thiolysis yield, but rather enhanced the degradation of the extracts. The likelihood of thiolysis resistant structures such as branched, substituted and oxidized tannins among the extracted phenolic oligomers was thus expected.

Carbohydrates in all the extracts were represented by both mono-/oligosaccharides (free carbohydrates) and bound carbohydrates, which can include both polysaccharides and glycosyl residues. In Silver fir, European larch and Douglas fir free carbohydrate were prevalent, while in Norway spruce and Scots pine bound carbohydrates represented the large majority (Table 7). Bound carbohydrate yields across the species varied limitedly across the

species (8.8 - 15.1 g/kgDB - Table 7). Similar results were reported in previous studies (Weissman 1985; Kempainen et al. 2014).

The results for the free carbohydrate yields significantly varied between 1.4 and 19.8 g/kg_{DB} (Table 7), a wide range that remained however below the concentration of monosaccharides reported by Weissmann (1984) and Krogell et al. (2012) for Norway spruce bark. A wide spread in the extraction yield was also detected in the phenolic yield of F1, corresponding to phenolic monomers and small oligomers (1.3 - 18.4 ECEg/kg_{DB}; Table 6). The greater extent of leaching of small molecular weight extractives from the bark of logs left exposed to atmospheric agents, as well as their easier biological degradation, more than the respective oligomers and polymers was reported (Field and Lettinga 1992). The storage history of the bark before collection could then be responsible for the observed variations in phenolic monomers and free carbohydrates as well as the intrinsic characteristics of the softwood species. This assumption is corroborated by observing that the species with lowest yields in free carbohydrates (Norway spruce and Scots pine) also had the lowest yields in phenolic monomers, while the highest free carbohydrates yield was measured for the species with the highest yield in phenolic monomers (Douglas fir). The changes in the yield and composition of bark extracts during prolonged outdoor storage of logs will be further discussed in section 4.3.4.

Table 7: Total, free and bound carbohydrates in softwood bark extracts (mean ± std.dev.; n = 1). Free carbohydrates are associated to mono- and oligosaccharides. Bound carbohydrates are associated to polysaccharides and glycosyl residues

	Total g/kg _{DB}	Carbohydrates Free g/kg _{DB}	Bound g/kg _{DB}
Silver fir	34.9 ± 0.4*	19.8 ± 0.2*	15.1 ± 0.2*
European larch	19.3 ± 0.3*	10.4 ± 0.2*	8.8 ± 0.2*
Norway spruce	16.5 ± 0.2*	2.1 ± 0.1*	14.4 ± 0.2*
Douglas fir	31.4 ± 0.2*	17.8 ± 0.2*	13.6 ± 0.2*
Scots pine	12.2 ± 0.1*	1.4 ± 0.1*	10.9 ± 0.1*

* estimated analytical error based of previous measurements
kg_{DB} = kg of dry bark

The hot water extracts from European softwood bark appear therefore to be a complex mixture of phenolic compounds and carbohydrates, which significantly vary across the species. Linear and non-oxidized condensed tannins appeared to represent only a minor fraction of the extracts. In contrast, the analysis on commercial samples of Black wattle and Quebracho extracts (data not reported) showed a scarce occurrence phenolic monomers and total carbohydrate concentrations equal to 6.8% and 9.0%, respectively, significantly lower

than in the studied species. Different chemical and physical characteristics of the European softwood bark extracts are therefore expected.

4.2.2 Phenolic monomers and other small molecular weight extractives

The analysis of the UV-absorption spectra (240-500 nm) and the mass spectra (200 - 700 m/z) in correspondence of the HPLC-UV chromatographic peaks of crude bark extracts (Figure 16) enabled identifying some of the extracted phenolic monomers. MALDI-TOF mass spectra of the crude extracts in the small mass range (300-900 m/z) were also examined to identify of molecular ions that matched with the molecular masses of typical softwood bark extractives (Bianchi et al. 2014).

In Norway spruce and European larch an HPLC-UV peak was observed at the retention time (RT) of 38.9 min (Figure 18 and Figure 19; compound "a"). In correspondence, the UV-spectrum showed two absorption maxima (λ_{MAX}) at around 290 and 320 nm, typical of compounds containing hydroxycinnamic groups (Bartolomé et al. 1993). The HPLC mass spectrum of the compound showed negative ions with mass equal to 405 m/z. The peak was therefore attributed to the stilbene glucoside astringin (MW = 406.4 Da; Figure 6). Accordingly, in the MALDI-TOF mass spectrum, molecular ions of 445.6 and 445.7 m/z were detected, that match with an astringin molecule combined with a potassium ion (K^+), which was used as cationization agent in the analysis ($406.4 + K^+ = 435.5$ Da). Piceid and isorhapontins (MW = 390.4 Da and 420.4 Da; Figure 6), two other stilbene glucosides, were similarly detected in Norway spruce HPLC chromatogram (RT = 43.1 min) and MALDI-TOF mass spectrum (429.7 and 459.6 m/z) (Figure 19; compounds "p" and "r", respectively).

Stilbenes glucosides and their aglycons are typical extractives of bark and knots from spruce species (Latva-Mäenpää et al. 2013), but were seldom identified in other softwoods. Their presence in the bark of larch species is suggested for the first time in this study.

In all species with the exception of Silver fir two HPLC-UV peaks at RT = 39.6 and 40.6 min were detected. The peak at RT = 39.6 min in Douglas fir chromatogram was particularly intense. Its absorption area was more than 20 times higher than in other species. Both peaks showed $\lambda_{MAX} = 290$ nm and no significant absorption in the range 300-500 nm, which is characteristic of flavonoids lacking conjugation with the aromatic B-ring. In correspondence, ion masses equal to 303 m/z were detected in the HPLC mass spectrum. The results match with the flavanone taxifolin (MW = 304.3 Da; Figure 6), most likely occurring as two different isomers (Figure 18 to Figure 21; compounds "t" and "t'"). However, none of the MALDI TOF mass spectra suggested the presence of taxifolin ($304.3 + K^+ = 343.4$ m/z).

The intense taxifolin peaks detected in Douglas fir chromatograms is in agreement with the relatively high amount of total phenolic monomers measured for this species by the

Folin-Ciocalteu assay (Table 6). Abundance of taxifolin in Douglas fir bark has been previously reported (Barton and Gardner 1958). Its detection in Norway spruce and other pine species has also been documented (Yazaki and Hillis 1977; Weissman 1981; Pan 1995; Yesil-Celitikas et al. 2009b).

Very low intense UV absorption peaks in the HPLC chromatograms at RT = 21.7 and 32.6 min corresponded, by comparison with the RT of analytical standards, to catechin and epicatechin monomers, respectively (Figure 17 to Figure 21; compounds “c” and “e”). Likewise, in Silver fir HPLC chromatogram (Figure 17) additional UV absorption peaks at RT = 12.5 and 21.1 min were detected and identified with galocatechin and epigallocatechin, respectively. For all species, the total concentrations of these flavan-3-ol were < 1.5% w/w of the extracts. Similar as observed for taxifolin, in the MALDI-TOF mass spectra no peaks could be detected that correspond to the (epi)catechin ($288.3 + K^+ = 329.4$ m/z) and (epi)galocatechin ($304.3 + K^+ = 345.4$ m/z) expected molecular ions. Mass peaks associated to (epi)catechin dimers and trimers ($578.6 + K^+ = 617.7$ m/z and $866.9 + K^+ = 906.0$ m/z) were instead detected in the MALDI-TOF mass spectra of European larch, Norway spruce, Douglas fir and Scots pine extracts (Figure 18 to Figure 21; compounds “2c” and “3c”).

Analogously, a mass peak that corresponded to a (epi)galocatechin dimer ($610.6 + K^+ = 649.7$ m/z) was observed in Silver fir (Figure 17; compounds “2g”). The Scots pine mass peak at 497.8 m/z (Figure 21; compound “gg” in) was attributed to galocatechin gallate (MW = 458.3; Figure 6).

Some peaks detected in the MALDI-TOF mass spectra were associated to non-phenolic extractives. Sucrose, stachyose, raffinose, pentose hexamers and hexose pentamers were attributed to the mass peaks at about 381.4 m/z ($= 342.3 + K^+$), 543.6 m/z ($= 504.5 + K^+$), 705.7 m/z ($= 666.6 + K^+$), 717.8 m/z ($= 678.7 + K^+$) and 849.9 m/z ($= 810.8 + K^+$) (Figure 17, Figure 19, Figure 20 and Figure 21; compounds “s1”, “s2”, “s3”, “s4” and “s5”). The mass peak at about 849.9 m/z was actually characterized by an ambiguous attribution, since it could alternatively be interpreted as an astringin dimer, which has been reported among Norway spruce bark extractives (Li et al. 2008; Zhang and Gellerstedt 2008).

Numerous HPLC and MALDI-TOF peaks remained unidentified. In particular, European larch chromatogram showed a very intense UV absorption peak at RT = 44 min, characterized by a $\lambda_{MAX} = 265$ nm and ionic mass equal to 284 m/z, which could not be attributed to any known bark extractive. The MALDI-TOF spectra of all the species showed intense peaks at 390 and 404 m/z, which also could not be attributed to any known substances, excluding the remote possibility of piceid and astringin ions without K^+ . All these non-identified compounds might be associated to lignans, less common flavonoids or, in the case of MALDI-TOF mass peaks, other non-phenolic substances (Pan 1995; Yesil-Celitikas et al. 2009; Jerez et al. 2009; Ucar et al. 2013).

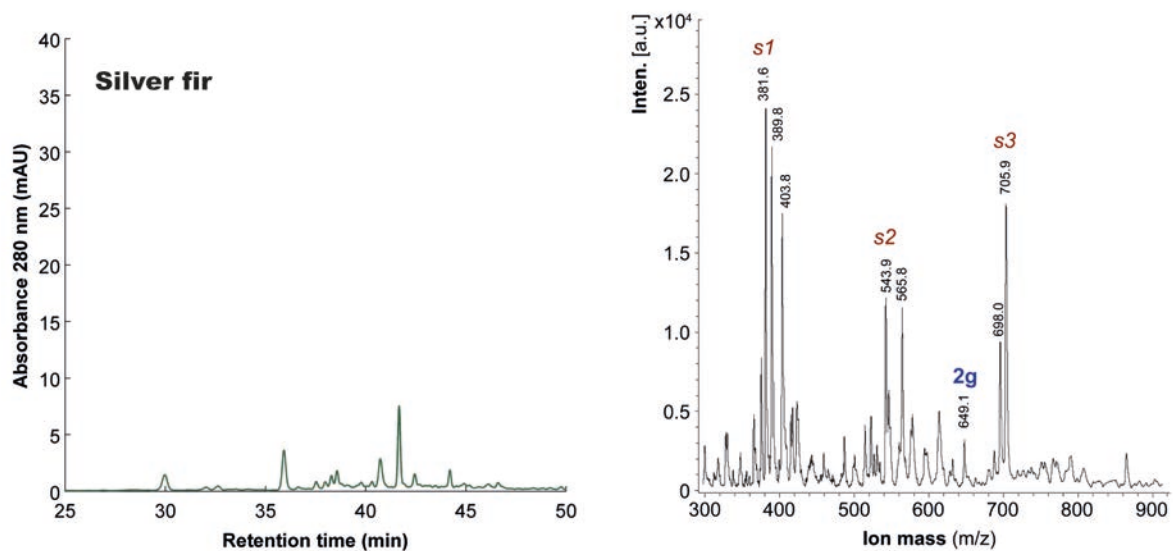


Figure 17: HPLC-UV chromatogram (25-50 min, left) and MALDI-TOF mass spectrum (300-900 m/z, right) of Silver fir bark crude extracts. The labelled peaks were identified as: 2g = (epi)gallocatechin dimer; s1 = sucrose; s2 = raffinose; s3 = stachyose.

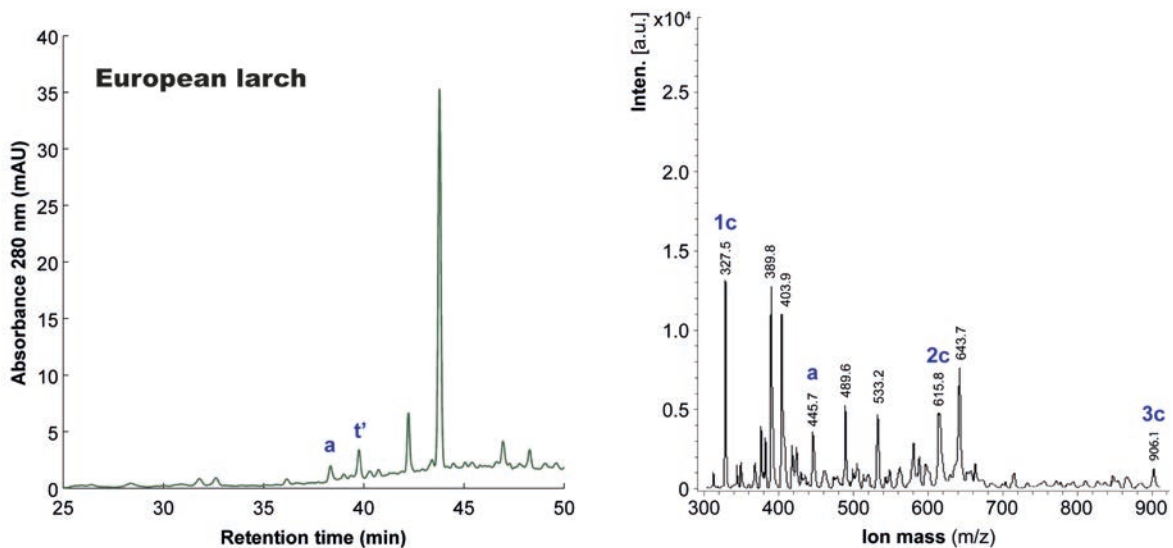


Figure 18: HPLC-UV chromatogram (25-50 min - left) and MALDI-TOF mass spectrum (300-900 m/z - right) of European larch bark crude extracts. The labelled peaks were identified as: a = astringin; t' = taxifolin isomer; 2c = (epi)catechin dimer; 3c = (epi)catechin trimer.

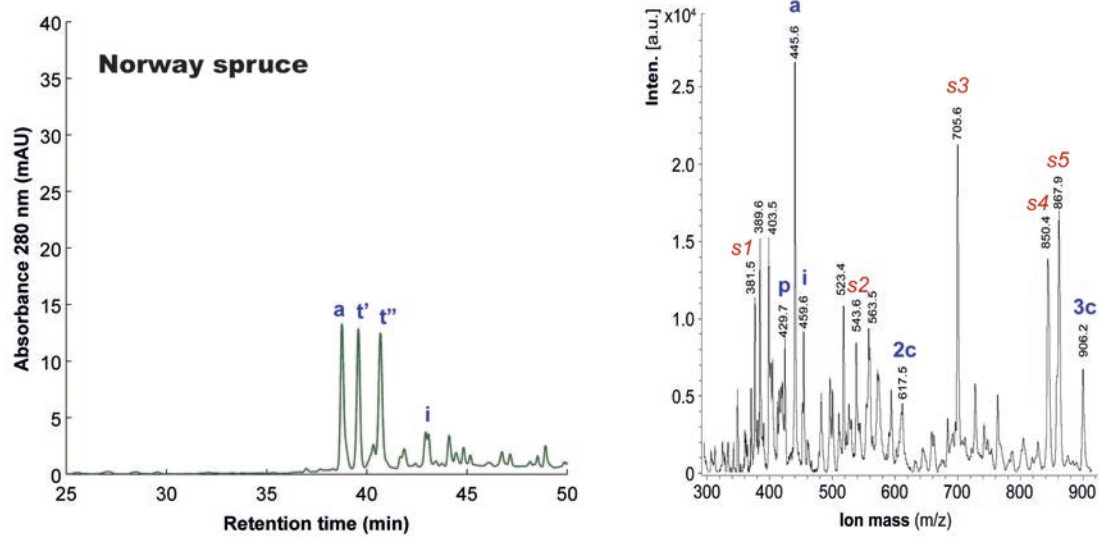


Figure 19: HPLC-UV chromatogram (25-50 min - left) and MALDI-TOF mass spectrum (300-900 m/z - right) of Norway spruce bark crude extracts. The labelled peaks were identified as: p = piceid; a = astringin; i = isorhapontin; t' and t'' = taxifolin isomers; 2c = (epi)catechin dimer; 3c = (epi)catechin trimer; s1 = sucrose; s2 = raffinose; s3 = stachyose; s4 = pentose hexamer; s5 = hexose pentamer.

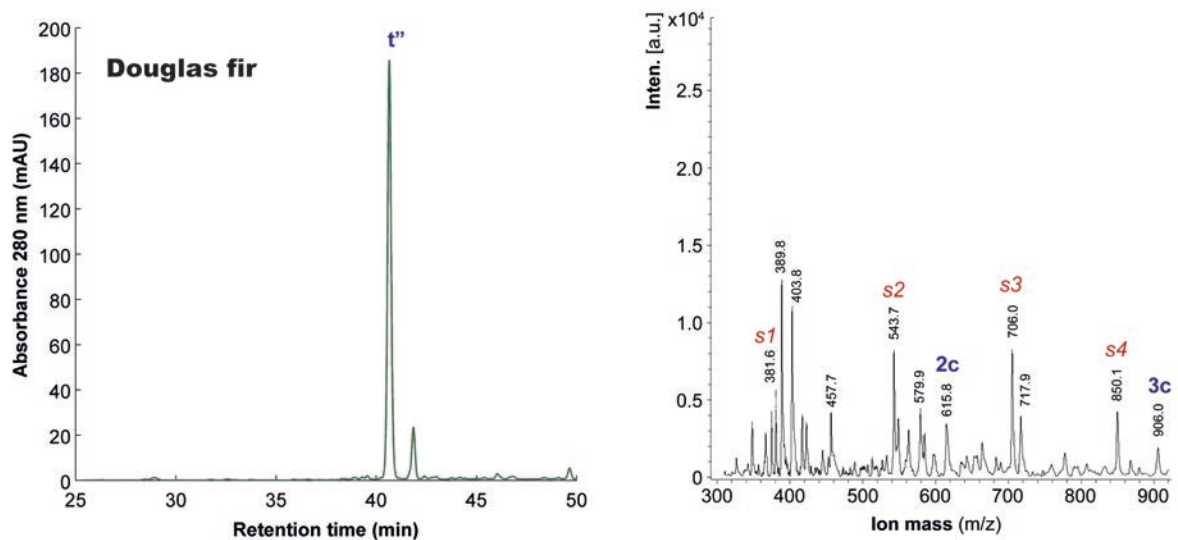


Figure 20: HPLC-UV chromatogram (25-50 min - left) and MALDI-TOF mass spectrum (300-900 m/z - right) of Douglas fir crude bark extracts. The labelled peaks were identified as: t'' = taxifolin isomer; 2c = (epi)catechin dimer; 3c = (epi)catechin trimer; s1 = sucrose; s2 = raffinose; s3 = stachyose; s4 = pentose hexamer.

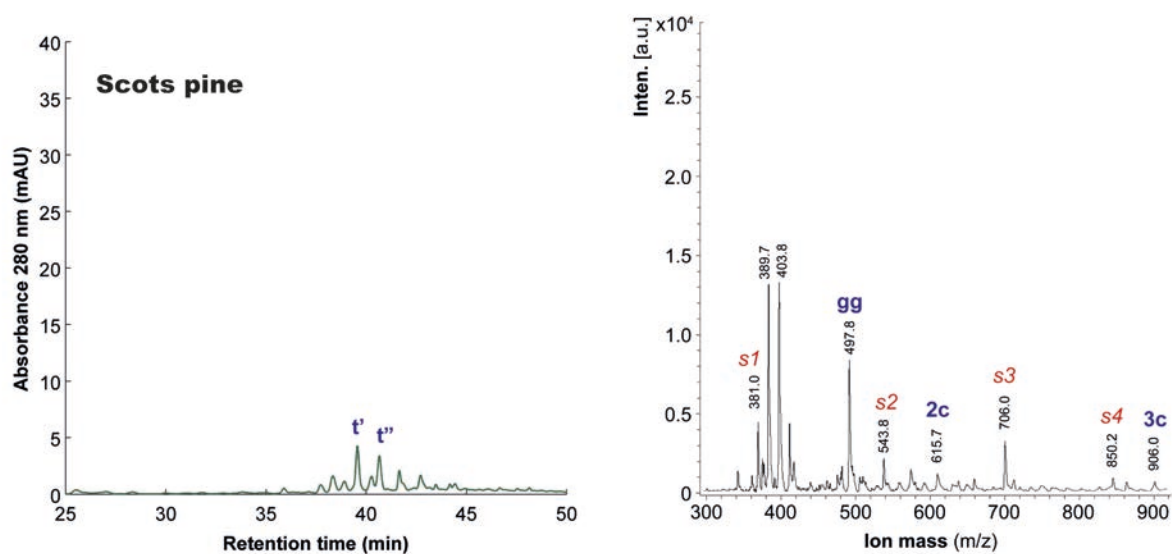


Figure 21: HPLC-UV chromatogram (25-50 min - left) and MALDI-TOF mass spectrum (300-900 m/z - right) of Scots pine crude bark extracts. The labelled peaks were identified as: t' and t'' = taxifolin isomers; gg = gallicocatechin gallate; 2c = (epi)catechin dimer; 3c = (epi)catechin trimer; s1 = sucrose; s2 = raffinose; s3 = stachyose; s4 = pentose hexamer

4.2.3 Molecular structure of the extracted condensed tannins

The presence of condensed tannins in the softwood bark extracts was shown by both reverse phase HPLC-UV after acid thiolysis and MALDI-TOF mass spectrometry (Bianchi et al. 2014; 2015). After thiolysis of the extracts, cleaved catechin, epicatechin, gallicocatechin, epigallocatechin and their thioethers were detected in the HPLC-UV chromatograms. Norway spruce, Douglas fir and Scots pine showed the exclusive presence of procyanidins (Table 8). In European larch, procyanidins with a 10% substitution of prodelphinidins units were detected. Prodelphinidins were however predominately present in Silver fir extracts, in agreement with the thiolysis results on milled bark of the same species (Table 3). Silver fir prodelphinidins showed 21% substitution with procyanidin units (Table 8). The higher hydroxylation of Silver fir and European larch tannins than tannins from other species could explain the low efficacy in SPE separation between phenolic monomers and polymers observed for these extracts (Table 6). Changes in the tannin polarities might in fact affect their affinities with solvents and solid phases.

In procyanidins, *cis*-configuration of the monomers was predominant, and *trans*-configurations almost restricted to the terminal units. In Silver fir both terminal and extension units of prodelphinidins displayed *trans*-configuration (Table 8). Analogous tannin

structures were shown in previous studies on softwood tannins (Porter 1989, Matthews et al. 1997b, Jerez et al. 2007). Thiolysis on the fraction F1 and F2 showed consistent molecular structures between shortest and longest tannins, with the exception of European larch. For this species in fact only the procyanidins eluted in the fraction F1 showed substitution with prodelphinidin units. The occurrence of prodelphinidins units in European larch tannin was therefore considered limited to the shortest oligomers.

Table 8: Structural characteristics determined by thiolysis and HPLC-UV of softwood bark condensed tannins in crude hot water extracts and extract fractions F1 and F2

Species	Crude extract			Fraction F1			Fraction F2		
	PC:PD %:%	<i>cis:trans</i> %:%	mDP	PC:PD %:%	<i>cis:trans</i> %:%	mDP	PC:PD %:%	<i>cis:trans</i> %:%	mDP
Silver fir	21:79	31:69	3.5	16:84	37:63	3.1	19:81	39:61	3.6
European larch	90:10	71:29	5.6	83:17	64:36	4.1	100:0	74:26	6.5
Norway spruce	100:0	76:24	6.2	100:0	67:33	3.5	100:0	79:21	8.2
Douglas fir	100:0	75:25	4.4	100:0	70:30	2.6	100:0	77:23	4.6
Scots pine	100:0	82:18	6.7	100:0	79:21	2.7	100:0	82:18	8.4

PC = procyanidin monomers (catechin, epicatechin)
 PD = prodelphinidin monomers (gallo catechin, epigallo catechin)
cis = *cis*-configured monomers (epicatechin, epigallo catechin)
trans = *trans*-configured monomers (catechin, gallo catechin)
 mDP = mean degree of polymerization

MALDI-TOF mass spectra of European larch, Norway spruce, Douglas fir and Scots pine in the range 900 - 4000 m/z (Figure 23 to Figure 26) showed the presence of a regular series of repeating peaks (EL1, NS1, DF1, SP1). In all these series the peaks were constantly separated from each other by about 288.3 m/z. This distance matches the mass of a procyanidin unit in a tannin oligomer ($290.3 - 2H = 288.3$ Da).

The MALDI-TOF mass spectra of Silver fir (Figure 22) showed instead a peak series (SF1) where the distance between the peaks was approximately 304.3 m/z, which matches the mass of a prodelphinidin unit ($306.4 - 2H = 304.3$ Da). These findings agreed with the thiolysis results, confirming the presence of procyanidins in all softwood bark extracts with the exception of Silver fir, for which prodelphinidins were detected. In Silver fir, a group of less intense peaks adjacent to the main peaks at distances equal to 16 m/z, or a multiple of it, was observed. This distance corresponded to the difference between a prodelphinidin and a procyanidin unit ($304.4 - 288.4 = 16.0$ Da). The frequent substitution of Silver fir prodelphinidin units with less hydroxylated procyanidin units, made already apparent in the thiolysis results (Table 8), was thus confirmed by the MALDI-TOF mass spectrum.

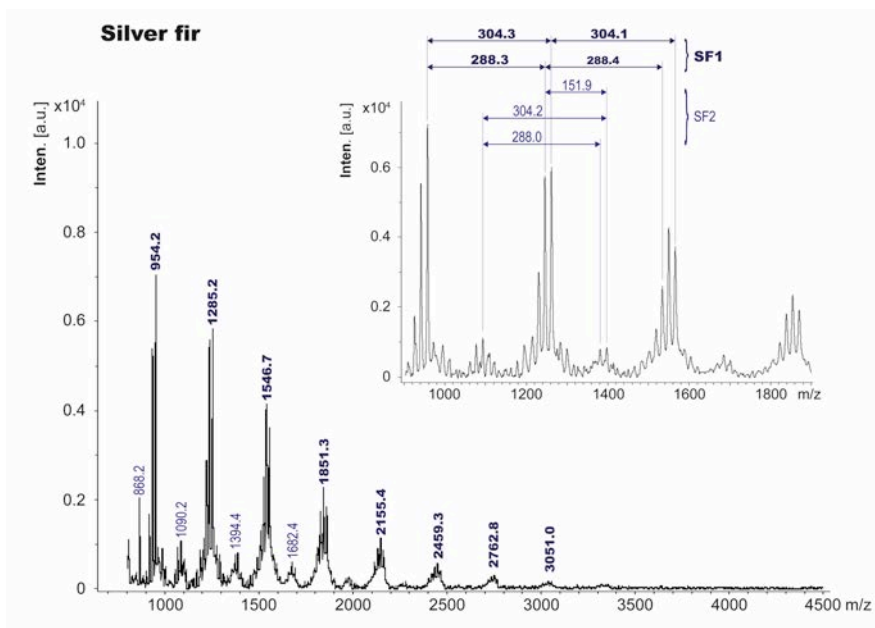


Figure 22: MALDI-TOF mass spectra (900-4000 m/z) of Silver fir bark extracts. The prodelphinidin series SF1 (**blue bold**) and SF2 (**blue narrow**) are apparent. The shift between them (≈ 152 m/z) is attributed to a gallate ester group (modified from Bianchi et al. 2014).

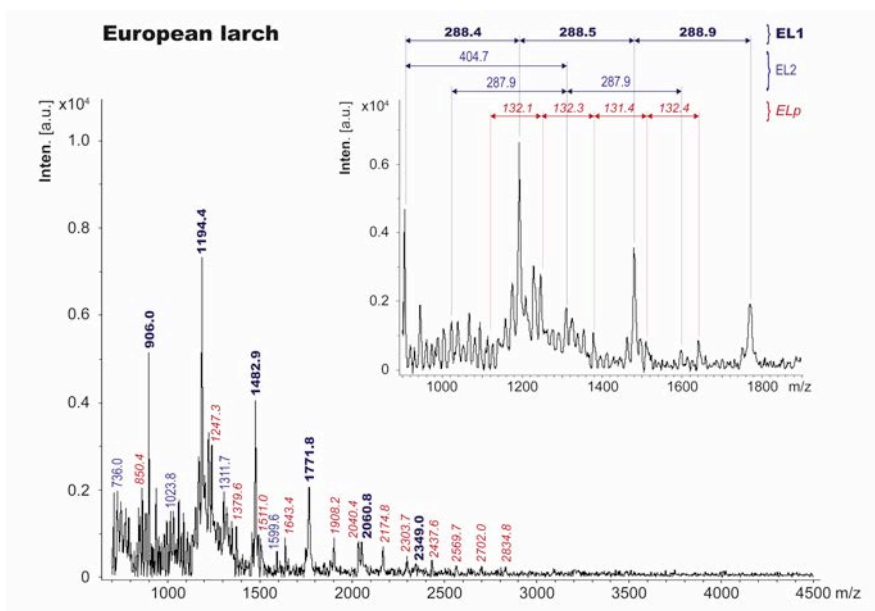


Figure 23: MALDI-TOF mass spectra (900-4000 m/z) of European larch bark extracts. The condensed tannin series EL1 (**blue bold**) and EL2 (**blue narrow**) are apparent. The shift between them (≈ 405 m/z) was associated to an astringin monomeric unit in the tannin structures. The series ELP (*red italic*) is identified as a polysaccharide made of pentose units (e.g. arabinan) (modified from Bianchi et al. 2015).

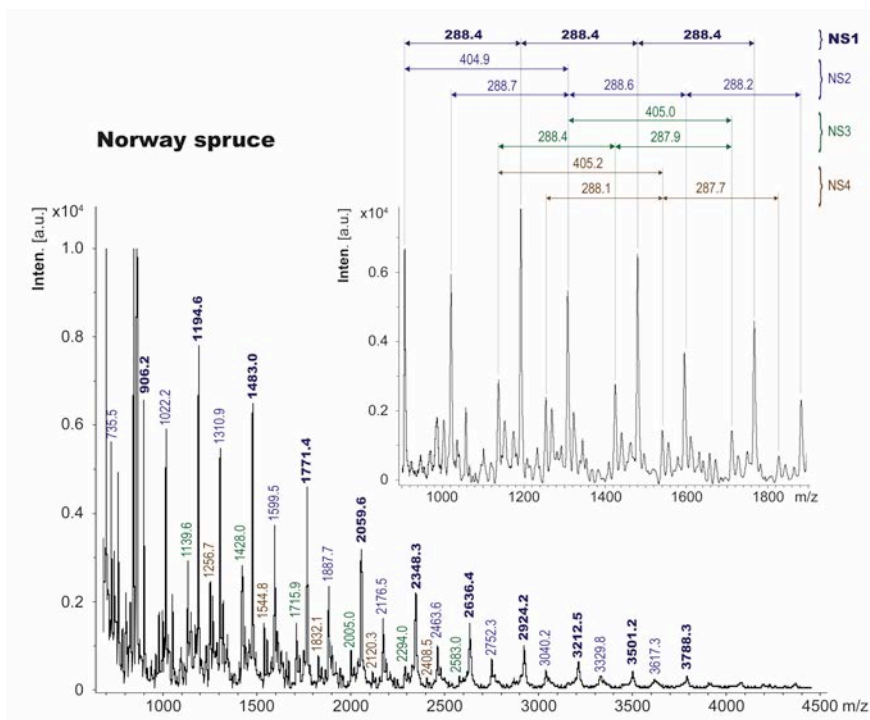


Figure 24 MALDI-TOF mass spectra (900-4000 m/z) of Norway spruce bark extracts. The condensed tannin series NS1 (**blue bold**), NS2 (**blue narrow**), NS3 (**green narrow**) and NS4 (**brown narrow**) are apparent. The shift between them (≈ 405 m/z) is attributed to astringin monomeric units in the tannin structure (modified from Bianchi et al. 2014).

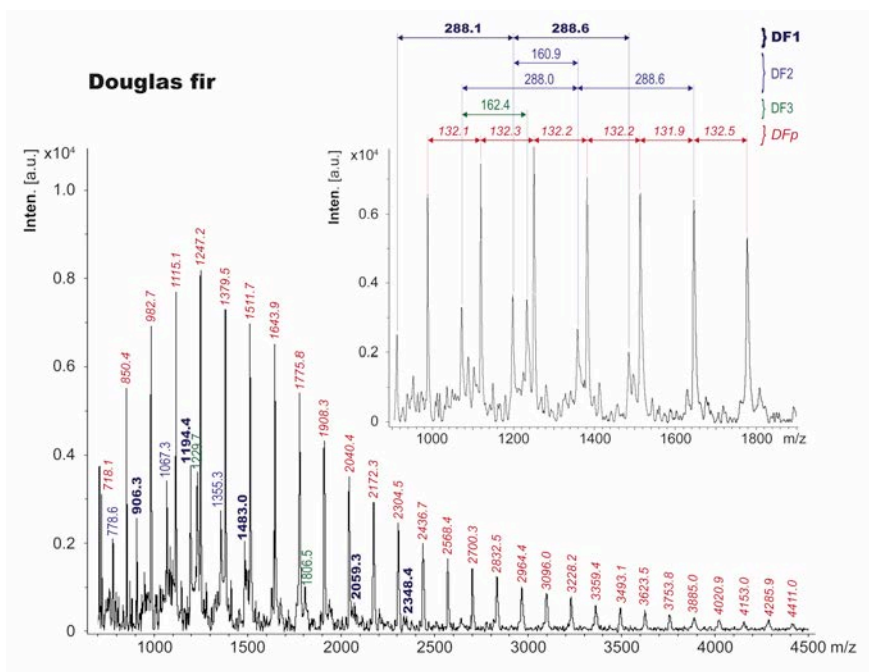


Figure 25: MALDI-TOF mass spectra (900-4000 m/z) of Douglas fir bark extracts. The condensed tannin series DF1 (**blue bold**), DF2 (**blue narrow**), DF3 (**green narrow**) are apparent. The shift between them (≈ 162 m/z) is attributed to glucoside groups. The dominant series DFP (**red italic**) is attributed to a polysaccharide made of pentose units (e.g. arabinan).

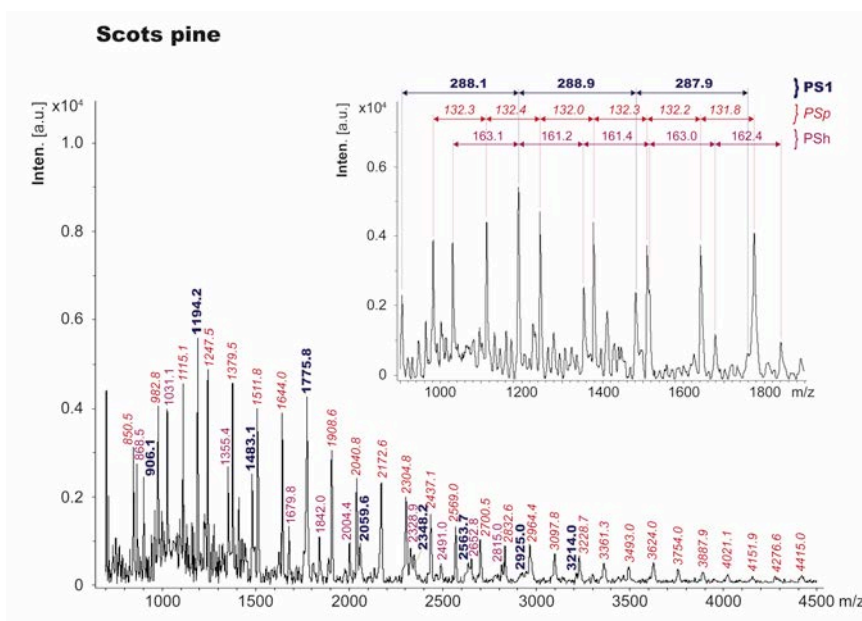


Figure 26: MALDI-TOF mass spectra (900-4000 m/z) of Scots pine bark extracts. The procyanidin series SP1 (**blue bold**) is apparent. The series PSp (*red italic*) and PSh (*purple regular*) are attributed to polysaccharides made of pentose (e.g. arabinan) and hexose (e.g. glucan) units, respectively (modified from Bianchi et al. 2015).

Each MALDI-TOF mass peak in the series SF1, EL1, NS1, DF1, SP1 (Figure 22 to Figure 26) could be interpreted with Equation 1:

$$M + K^+ = x \cdot 288.3 + y \cdot 304.3 + 2.0 + 39.1 \quad (1)$$

where M is the mass of the tannin oligomer, x and y are the numbers of procyanidin and prodelphinidin units in the oligomer, respectively, 2.0 is the mass of the two terminal hydrogens, and 39.1 is the mass of the cationization agent (K^+).

The equation permitted to estimate the maximum degree of polymerization of the extracted tannin, which corresponded to the sum of x and y for the last observable peak in the mass spectra. In detail, Silver fir, European larch, Norway spruce, Douglas fir and Scots pine bark extracts showed maximum polymerization degrees equal to 10, 8, 13, 8 and 11 units, respectively.

In addition to the main procyanidin or prodelphinidin series, most of the MALDI-TOF mass spectra displayed secondary peak series with the same repeating unit of 288.3 or 304.3 m/z, but upscale shifted from the main peaks. They were interpreted with the occurrence of monomers other than flavan-3-ols within some tannin oligomers. More specifically, in Silver fir a low intense prodelphinidin series shifted of about 152 m/z was detected (SF2; Figure 22). The shift was associated to the occasional presence of a galloyl moiety (152.1 Da) in the tannins, e.g. a gallo catechin gallate unit (Bianchi et al., 2014).

In European larch and Norway spruce mass spectra, a procyanidins peak series shifted of about 405 m/z was instead observed (EL2 and NS2; Figure 23 and Figure 24). The presence within the procyanidin molecular structure of an astringin unit (406.4 - 2H = 404.4 Da) was supposed (Bianchi et al., 2014; 2015). The occurrence of stilbene units in the bark tannins of spruce species, but not for larch, has already been suggested by different authors (Hergert, 1989; Matthews et al., 1997a; Zhang and Gellerstedt, 2008). A detailed analysis of the Norway spruce MALDI-TOF mass spectrum even showed the presence of peaks that could be attributed to the presence of 2 or 3 astringin units in the tannin oligomers (series NS3 and NS4; Figure 24). The peaks at 850.4 and 1256.6 m/z could be actually identified as astringin dimers and trimers (Bianchi et al., 2014). Astringin monomer, dimers and trimers could then be most likely regarded as terminal units to which regular procyanidin oligomers are linked, in agreement with the 2D-NMR spectra interpretations of Zhang and Gellerstedt (2008) on isolated Norway spruce bark tannins. The likely molecular structure of Norway spruce bark tannins is schematically presented in Figure 27.

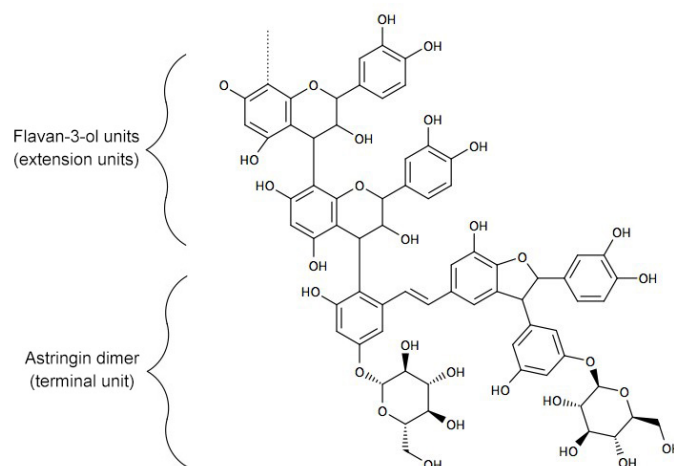


Figure 27: Likely structure of a Norway spruce bark tannin containing an astringin dimer as a terminal unit.

The intensity of the mass peaks relative to tannins containing an astringin unit in Norway spruce mass spectrum (series NS2; Figure 24) is only slightly lower than the main peak series (NS1). In European larch, the intensity of the EL2 series is instead much lower than the EL1 series (Figure 23). A more frequent occurrence of astringin units in Norway spruce tannins than European larch was thus hinted. The presence of stilbene glucosides in the tannins of European larch and Norway spruce could be correlated to the low thiolysis yield observed for these species. The link between stilbenes and flavan-3-ols has in fact been recognized as thiolysis resistant (Matthews et al. 1997a).

Douglas fir MALDI-TOF spectrum showed a secondary peak series shift from the main one of 162 m/z, or a multiple of it (DF2 and DF3; Figure 25). The likelihood of glucosylation ($180.2 - \text{H}_2\text{O} = 162.2 \text{ Da}$), e.g. occurrence of epicatechin glucoside units, along the tannin oligomers was indicated (Bianchi et al., 2015). The low thiolysis yield also observed for this species (Table 6) could not be clearly associated to the presence of links with monomers other than flavan-3-ols. The occurrence of highly branched oligomers, which are more thiolysis resistant than linear oligomers (Hemingway and McGraw 1983) and were described by Foo and Karchesy (1989d) in Douglas fir bark extracts, was thus supposed.

The mDP of the extracted softwood tannins varied from 3.5 (Silver fir) to 6.7 (Scots pine) (Table 8). These values were only slightly lower than those detected for total tannins after thiolysis of the bark (Table 3). The degree of polymerization of the tannins therefore seemed to barely influence their extractability. The presence of intermolecular bonds among tannins and other bark constituents was consequently considered as the major extraction constraint, as already suggested by Matthews et al. (1997a).

The identified molecular structure of the analyzed European softwood bark tannins considerably differ from Black wattle and quebracho tannins, which are mostly represented by fisetinidins and robinetinins with an average polymerization degree between 3 and 4 (Pizzi and Stephanou 1994b; Pasch et al. 2001; Venter et al. 2012a, 2012b). Difference stereoconfiguration of the flavan-3-ol units were also observed in comparison with the more investigated Radiata pine tannins (Matthews et al. 1997a; Jerez et al. 2009a), which most likely results in an opposite orientation of the tannin oligomer helix (Zucker 1983). In combination with the frequent substitution of flavan-3-ol units with stilbenes, recurrent glucosylation and extensive oxidation and/or rearrangement, these characteristics most likely influence the chemical reactivity of the European softwood bark tannins, which should therefore be considered substantially different from the currently used products.

4.2.4 Monomeric composition of extracted carbohydrates

Glucose and fructose were the main extracted monosaccharides in all species (Table 9). Oligosaccharides were detected only in traces with the exception of Silver fir extracts, in which sucrose was present in a comparable amount to glucose and fructose (Table 9).

Bound carbohydrates showed glucose, galactose and arabinose as main monomeric residues. Mannose and galacturonic acid were also detected but in minor concentrations (Table 9). This monomeric composition suggested that polysaccharides in softwood bark extracts were mainly represented by pectins such as arabinans, arabinogalactans and glucans (Timell 1961; Fu and Timell 1972a, 1972b; Le Normand et al. 2014). Mannose probably derived from galactoglucomannan fragments, a typical component of softwood bark hemicellulose (Timell 1961; Dietrichs et al. 1978). Extraction of arabinogalactan protein

complexes (AGP) was also considered. Elemental analysis indicated the occurrence of nitrogen between 0.28% and 0.52% of the dry extracts. Using a conversion factor equal to 6.25, a concentration of proteins in the extracts between 1.8% and 3.4% was therefore expected. Considering that the weight ratio between proteins and arabinogalactan in AGP is about 10:90 (Putoczki et al. 2007), the extracted proteins would be more than sufficient for the complexation of all the arabinogalactans in the extracts. Further and more specific investigations are needed to confirm the presence of AGP in the extracts.

Table 9: Monomeric composition of carbohydrates in softwood bark crude extracts and extract fractions F1 and F2. Free carbohydrates are associated to mono- and oligosaccharides. Bound carbohydrates are associated to polysaccharides and glycosyl residues. (n = 1).

			Carbohydrates (g/kg _{DB})								
			Glc	Gal	Fru	Man	Ara	GalA	Suc	Raf	Sta
Silver Fir	free	Total	4.00	<0.01	5.62	<0.01	<0.01	<0.01	8.08	1.06	1.25
	bound	Total	8.84	2.06	<0.01	0.42	1.56	0.69	-	-	-
		F1	1.77	0.04	<0.01	<0.01	0.19	-	-	-	-
		F2	1.22	0.10	0.02	0.07	0.65	-	-	-	-
European larch	free	Total	3.47	0.29	3.23	0.03	1.19	0.24	1.22	0.20	0.54
	bound	Total	3.88	0.44	<0.01	0.53	3.38	0.17	-	-	-
		F1	1.24	0.04	<0.01	0.01	0.15	-	-	-	-
		F2	1.43	0.18	<0.01	0.11	2.01	-	-	-	-
Picea abies	free	Total	0.63	0.06	0.68	0.01	0.16	0.11	0.38	0.07	<0.01
	bound	Total	8.21	1.46	<0.01	0.41	2.80	0.72	-	-	-
		F1	2.00	0.02	<0.01	<0.01	0.09	-	-	-	-
		F2	3.04	0.13	<0.01	0.09	1.27	-	-	-	-
Douglas fir	free	Total	9.95	0.15	5.23	0.03	0.84	<0.01	0.41	0.38	0.44
	bound	Total	4.10	2.03	<0.01	0.38	5.78	0.64	-	-	-
		F1	1.00	0.03	<0.01	0.01	0.07	-	-	-	-
		F2	0.77	0.29	<0.01	0.08	2.84	-	-	-	-
Scots pine	free	Total	0.97	0.01	0.20	<0.01	0.08	<0.01	0.08	<0.01	<0.01
	bound	Total	2.67	1.66	0.39	0.78	3.97	0.65	-	-	-
		F1	0.76	0.03	<0.01	0.01	0.22	-	-	-	-
		F2	0.49	0.16	<0.01	0.09	1.52	-	-	-	-

kg_{DB} = kg of dry bark

The hot water extraction of polysaccharides was also indicated by the MALDI-TOF mass spectra of Douglas fir and Scots pine extracts (Figure 25 and Figure 26, respectively). In these species the ratio between bound carbohydrates and phenolic oligomers was considerably higher than in other species (Table 6 and Table 7). Accordingly, the dominant mass peak series in the MALDI-TOF spectra (DFp and SPp) were not those of tannins, but a series that had a repeating unit of about 132 m/z. This value matches with the monomeric unit of a pentosan, e.g. arabinans. Traces of this series were also detected in the MALDI-TOF

spectrum of European larch (ELp, Figure 23). In Scots pine spectra (Figure 26), an additional peak series with a repeating unit equal to 162 m/z was attributed to a hexosan, e.g. glucosan (SPh).

The contribution of phenolic glycosyls to bound carbohydrates was evaluated through hydrolysis of fractions F1 and F2 (Table 9). The considerable presence of glucose residues in both fractions of all species suggested the presence of glucosylated phenolic monomers and oligomers in all softwood bark extractives. The comparison between the amounts of extracted phenolic compounds in F1 and F2 (Table 6) and the corresponding glucose residue yields (Table 9) suggested that between 9% and 22% of the phenolic monomers and oligomers in Silver fir, European larch, and Douglas fir were glucosylated. A much higher frequency of glycosylation was suggested for Norway spruce and Scots pine. In the F1 fractions, containing the phenolic monomer elution, 61% and 104% of glycosylated phenolics were estimated for Norway spruce and Scots pine, respectively. Oligomers in the fraction F2 of the same species showed 34% and 39% of glycosylation. The presence of astringin in Norway spruce bark extracts, both as free monomer and as monomeric units within the condensed tannins, was associated to the high glucose to phenolic ratio for this species. In Scots pine no evidence of phenolic glucosides was found by HPLC or MALDI-TOF mass spectrometry. The concentration of phenolic compounds and glucose in the extracts of this species was however very low, and the occurrence of phenolic glucosides might then have been below the detection limit.

In the fraction F2 of all species except Silver fir a considerable amount of arabinose residues was detected. Phenolic arabinosides are compounds scarcely detected among plant extractives, therefore their presence in bark extracts was considered unlikely. Arabinans are instead typical polyoses from softwood barks and quite soluble in alcohols (Fu and Timell 1972a; Hara et al. 2013). Their elution with methanol in the fraction F2 together with phenolic oligomers was thus considered possible. Accordingly, MALDI-TOF mass spectra of the F2 fractions of European larch, Douglas fir and Scots pine showed the presence of a pentosan series identical to that observed in the crude extracts (data not reported).

4.3 Analysis and optimization of the softwood bark extraction process

4.3.1 Extractions at different bark particle sizes

Water extractions at 60°C were performed on Norway spruce bark manually chopped (5-10 mm), ground with a hammer-mill (0.5-2 mm) or reduced to a fine powder with a vibratory disk mill (<0.05 mm). Higher extraction yields were shown at decreasing bark particle size (Figure 28), in agreement with Yazaki (1985) and Vélazquez et al. (2001). SEM

pictures of the different bark particles (Figure 29) indicate that the cellular structure of the bark was only recognizable in the chopped sample. The hammer-milled bark had extensively wrinkled and broken cell walls, while only fragments and flakes were observed in the bark subjected to the vibratory disk mill. Increased accessibility to bark cell walls and lumens was apparent as particle size decreased. Diffusion of extractives through the cell walls therefore seemed to represent a critical factor in the extraction process.

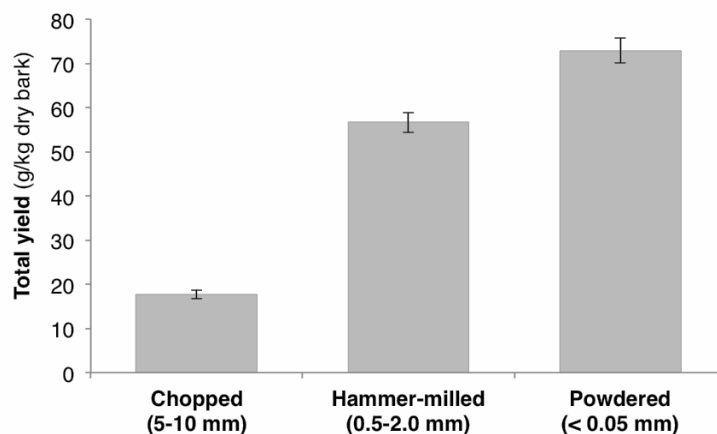


Figure 28: Total extraction yield of Norway spruce bark ground to different sizes. Extractions performed in water at 60°C for 10 min.

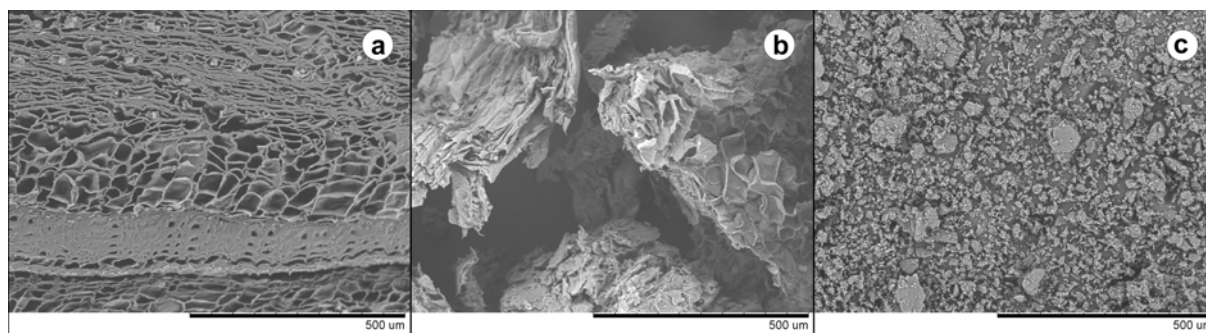


Figure 29: SEM pictures of Norway spruce bark ground to different size: a) manually chopped (5-10 mm); b) hammer-mill ground (0.5-2.0 mm); c) vibratory disk fine-milled (<0.05 mm).

To investigate the accessibility of bark deposits during extraction, UV-absorbance spectra of phenolic deposits at different depths from the surface were measured by UMSP in Norway spruce bark samples before and after extraction at 90°C (Bianchi et al. 2016). A clear reduction in the UV-absorbance spectrum was only observed for cork cell deposits at the outermost bark surface (Figure 30). The absorbance of sclereid deposits adjacent to the cork cell layer slightly decreased, and that of sclereids at approximately 200 µm in depth was only marginally affected by the extraction. No changes were observed in the absorbance

of cell walls before and after the extraction. The results revealed that the efficacy of the hot water extraction of phenolic compounds was limited to a few cell layers, as already pointed out by Shen et al. (1986) for Dahurian larch bark. Fine milling of the bark and breaking of its cellular structure were therefore considered important for the maximization of the extraction yield.

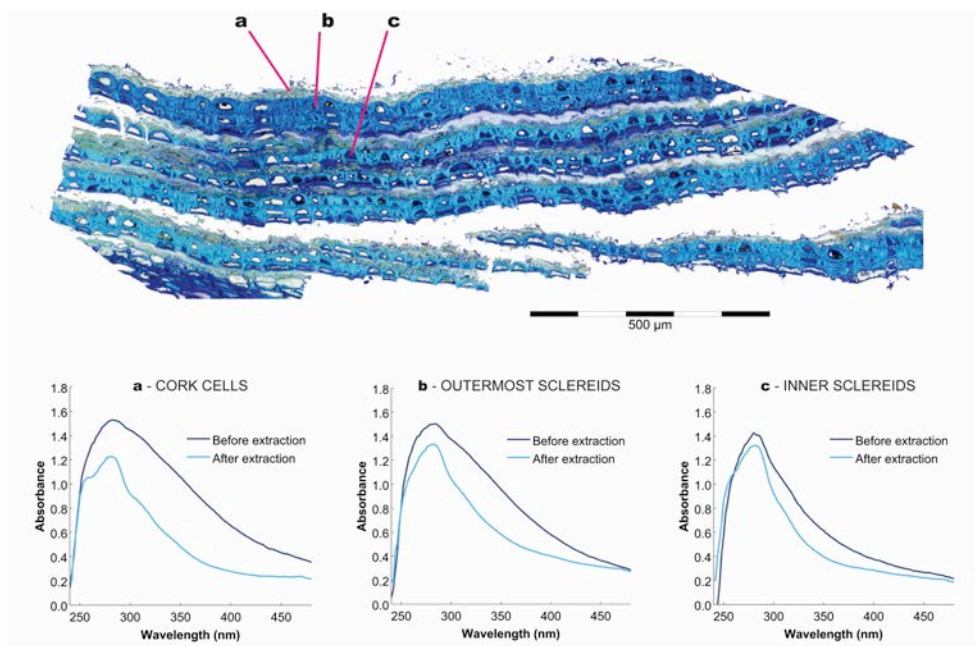


Figure 30: UV-absorbance spectra of phenolic deposits of Norway spruce bark at different depths from the surface (average of $n \geq 10$ measurements). The indicative position of the measurements is indicated on the above light microscope image (modified from Bianchi et al. 2016).

4.3.2 Response surfaces analysis of bark hot water extraction

The individual and combined effects of extraction temperature, extraction time, addition of sulfites and addition of urea to the extraction water were analyzed on Norway spruce bark applying the response surface method (Myers et al. 2009, **Bianchi et al. 2016**).

The extraction process factors were varied within the following ranges:

- Temperature (x_1) = 30 – 90 °C
- Time (x_2) = 2 – 60 min
- Sulfites, as SO_3^{2-} (x_3) = 0 – 5 g/L
- Urea (x_4) = 0 – 5 g/L

A face centered cubic experimental design was chosen. A total of 27 extraction runs (24 points + 3 central point repetitions) were performed in a random order. Total, phenolic, tannin and carbohydrate yields were measured for each extraction run (Table 10). In the presence of sulfites the standard Folin-Ciocalteu assay overestimates the concentration of

phenolic compounds and therefore couldn't be applied (Singelton et al. 1999). Therefore, the phenolic yield was estimated by measuring the absorbance at 280 nm of 0.1 g/L of aqueous solutions of extracts. Likewise, the tannin yield was estimated by applying the methylcellulose precipitation method (Sarneckis et al. 2006). Carbohydrates were quantified as the sum of the monomeric residues after acid hydrolysis of the extracts. The results were processed with the software Unscrambler-X (CAMO, Norway) and fitted using a least-square regression to a full quadratic model (Eq. 2).

$$Y = \beta_0 + \sum_{k=1}^4 \beta_k \cdot x_k + \sum_{j,k=1}^4 \beta_{jk} \cdot x_j x_k \quad (2)$$

For all yields, the analysis of the variance (ANOVA) showed an insignificant ($p > 0.150$) lack of fit (Table 11). The full quadratic model thus proved suitable for the description of the yield variations in the analyzed ranges.

Table 10: Experimental design performed on the hot water extraction of Norway spruce bark

Standard order	Random order	Temp. °C	Time min	Sulfites g/L	Urea g/L	Total yield g/kg _{DB}	Phenolic yield ECEg/kg _{DB}	Tannin yield ECEg/kg _{DB}	Carbohydrate yield g/kg _{DB}
1	24	30	2	0	0	13.2	7.4	4.0	4.0
2	20	90	2	0	0	35.4	28.5	18.2	9.0
3	14	30	60	0	0	18.8	10.2	5.6	5.6
4	9	90	60	0	0	44.3	34.0	22.0	14.3
5	1	30	2	5	0	55.9	7.4	3.1	4.7
6	22	90	2	5	0	88.2	28.3	17.8	8.2
7	19	30	60	5	0	65.9	8.3	2.6	5.2
8	15	90	60	5	0	119.8	57.8	33.9	17.6
9	11	30	2	0	5	55.6	9.5	5.0	4.0
10	8	90	2	0	5	80.6	29.9	19.3	8.4
11	16	30	60	0	5	52.0	9.0	4.8	4.2
12	27	90	60	0	5	93.8	38.5	24.8	14.2
13	13	30	2	5	5	103.4	8.6	3.2	4.6
14	12	90	2	5	5	132.3	30.1	16.7	9.3
15	2	30	60	5	5	102.9	10.5	4.2	6.1
16	26	90	60	5	5	169.4	55.6	33.8	18.5
17	25	30	31	2.5	2.5	61.5	8.9	4.3	5.1
18	10	90	31	2.5	2.5	92.0	40.6	26.7	12.6
19	18	60	2	2.5	2.5	71.9	17.6	9.5	6.2
20	7	60	60	2.5	2.5	82.8	23.6	14.6	8.9
21	4	60	31	0	2.5	50.0	20.3	13.2	5.0
22	3	60	31	5	2.5	106.9	22.0	11.3	8.5
23	5	60	31	2.5	0	56.4	22.0	13.6	7.9
24	17	60	31	2.5	5	93.0	21.7	11.9	7.5
25	6	60	31	2.5	2.5	75.3	19.6	11.1	8.9
26	23	60	31	2.5	2.5	78.5	20.8	10.7	7.3
27	21	60	31	2.5	2.5	73.8	20.9	12.7	7.8

ECEg = g (-)-epicatechin equivalent
kg_{DB} = kg of dry bark

Table 11: Significances (p-value) and regression coefficients (β) of the experimental design performed on hot water extraction of Norway spruce bark

	Total yield		Phenolic yield		Tannin yield		Carbohydrate yield	
	p-value	β	p-value	β	p-value	β	p-value	β
Intercept	<0.001 ***	76.4	<0.001 ***	21.16	<0.001 ***	12.37	<0.001 ***	7.64
Temperature (x_1)	<0.001 ***	18.1	<0.001 ***	14.63	<0.001 ***	9.79	<0.001 ***	3.81
Time (x_2)	<0.001 ***	6.3	<0.001 ***	4.47	<0.001 ***	2.50	<0.001 ***	2.00
Sulfites (x_3)	<0.001 ***	27.8	0.013 *	2.28	0.321	0.55	0.003 **	0.79
Urea (x_4)	<0.001 ***	21.4	0.509	0.53	0.767	0.16	0.963	0.01
$x_1 \cdot x_2$	<0.001 ***	5.0	<0.001 ***	4.00	<0.001 ***	2.54	<0.001 ***	1.60
$x_1 \cdot x_3$	0.002 **	4.2	0.008 **	2.65	0.021 *	1.50	0.209	0.30
$x_1 \cdot x_4$	0.117	1.8	0.930	0.07	0.935	0.05	0.616	0.11
$x_2 \cdot x_3$	0.007 **	3.4	0.009 **	2.59	0.025 *	1.45	0.051	0.48
$x_2 \cdot x_4$	0.564	-0.6	0.813	-0.20	0.782	0.16	0.993	0.00
$x_3 \cdot x_4$	0.640	0.5	0.783	-0.23	0.690	-0.23	0.213	0.29
x_1^2	0.968	0.1	0.142	3.24	0.078	2.72	0.030 *	1.37
x_2^2	0.805	0.7	0.650	-0.96	0.607	-0.75	0.878	0.09
x_3^2	0.504	1.8	0.849	-0.40	0.707	-0.54	0.214	-0.73
x_4^2	0.460	-2.0	0.867	0.35	0.976	-0.04	0.683	0.23
Lack of fit	0.241	--	0.163	--	0.177	--	0.497	--

*** p < 0.001 highly significant
 ** 0.001 ≤ p < 0.01 significant
 * 0.01 ≤ p < 0.05 poorly significant
 p ≥ 0.05 not significant

Extraction temperature, time and their interactions were the most significant factors in all yields (Table 11). The effect of the temperature was largely predominant, and particularly in the extraction of the phenolic compounds (Figure 31). For instance, a temperature increase from 30 to 90°C enabled, at 60 minutes extraction time and in pure water, a five-fold increase of total phenolic and tannin yields and a three-fold increase of carbohydrate yield. At shorter extraction times, the temperature was less effective. The extraction time didn't show any influence at the lowest temperatures, but gradually intensified its effect as the temperature increased (Figure 31). At 90°C, a gain of about 25% in the phenolic and tannin extraction yields was observed as extraction time increased from 2 to 60 minutes. Similar results were observed for the carbohydrate yield. The results were in good agreement with similar studies performed on Maritime pine and Norway spruce extractions (Jorge et al. 1999; Vazquez et al. 2001; Kemppainen et al. 2014).

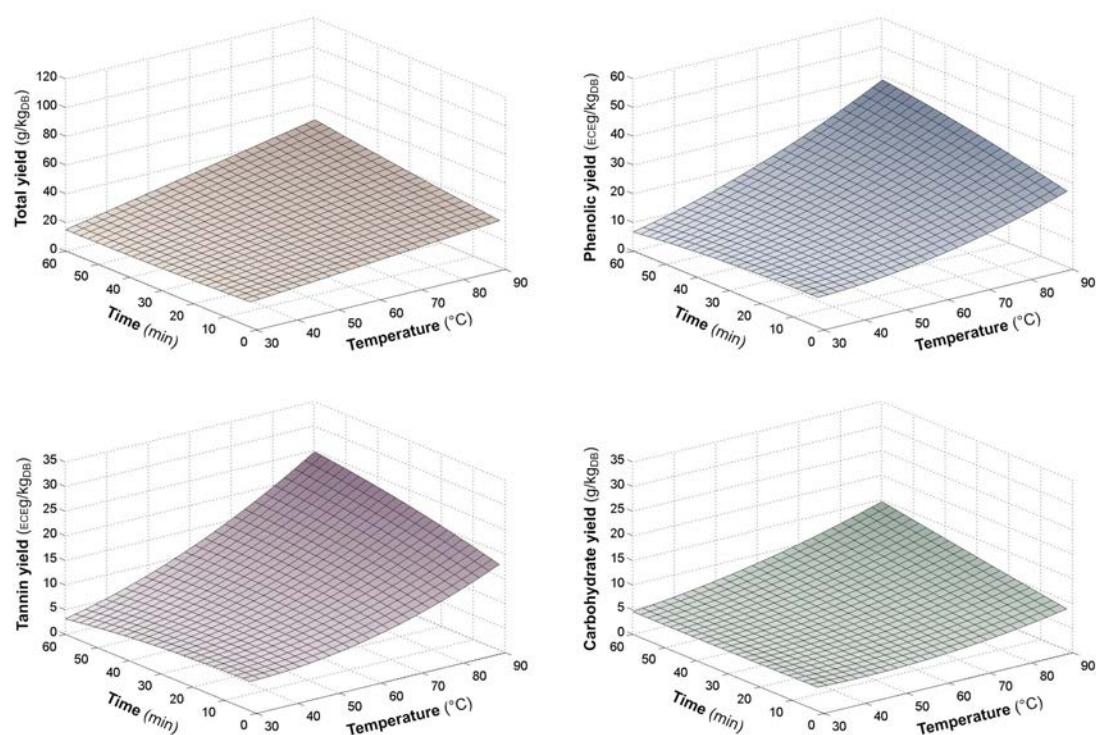


Figure 31: Response surface plots for total, phenolic, tannin and carbohydrate yields as a function of extraction temperature and time (sulfites = 0 g/L; urea = 0 g/L). Phenolic and tannin are expressed as epicatechin equivalents (ECEG). All yields are referred to in kg of dry bark (kg_{DB}) (modified from Bianchi et al. 2016).

The addition of sulfite and urea were both highly significant for the total yield (Table 11). The dramatic effect of sulfite on the total extraction yield is shown in Figure 32. A similar surface was obtained for urea (not reported). Phenolic, tannin and carbohydrate yields were instead slightly affected by the addition of sulfite (Figure 32), and even less by urea. The residual presence of sodium sulfite and urea in the dry extracts was considered the main reason for the observed total extraction yield increase, as already observed by Jorge et al. (1999). The improvement in the recovery of actual bark extractives was very limited. Data reported by Sealy-Fisher and Pizzi (1992) and König and Roffael (2003) concerning softwood bark extractions with and without sulfite and/or urea, resulted in similar outcomes when a mass balance was applied on the process. Subtle positive effects of sulfite on the phenolic and tannin yields could be detected only at the longest extraction times and highest temperatures (Figure 32), as suggested by the significance of the interaction factors $x_1 \cdot x_3$ ($p = 0.008$) and $x_2 \cdot x_3$ ($p = 0.009$) (Table 11). Such effects were not observed for urea. The significance of sulfite addition in the carbohydrate yields was most likely correlated to the high concentration of phenolic glucosides in Norway spruce bark extracts. Bertaud et al. (2012) detected a much higher impact of the sulfite and urea addition on the phenolic yield of softwood bark extraction, even if the process conditions

were very similar to the present study. Possible differences in the preparation and storage of the bark before extraction were supposed.

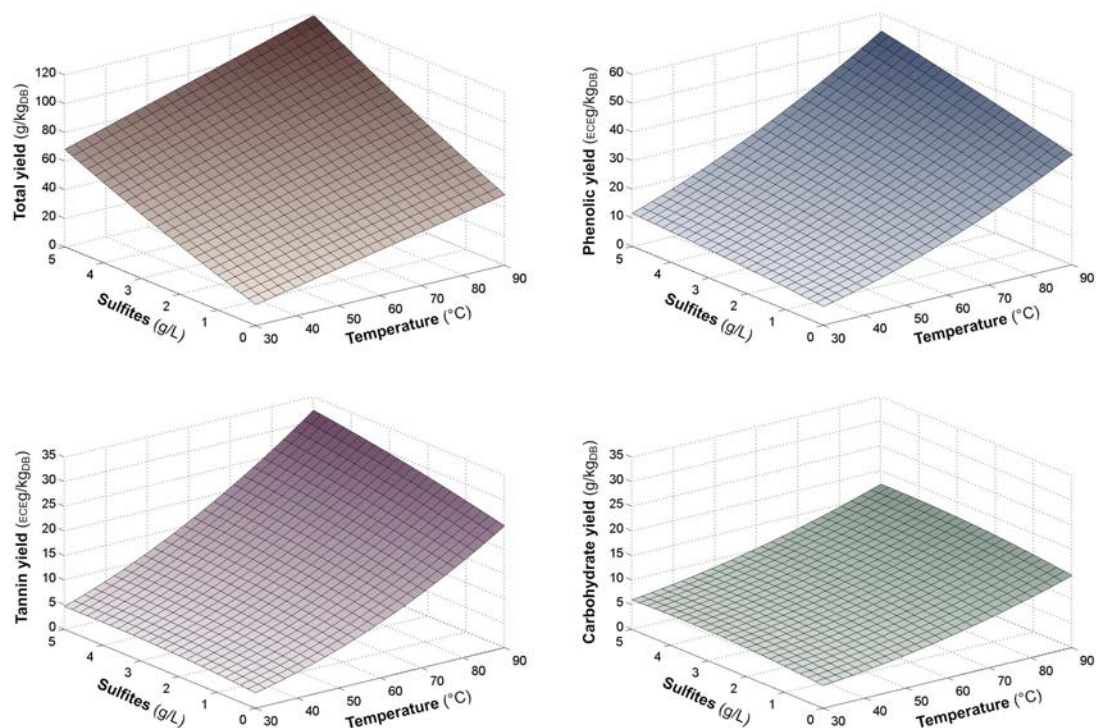


Figure 32: Response surface plots for total, phenolic, tannin and carbohydrate yields as a function of extraction temperature and concentration of sulfites in the extraction water (extraction time = 60 min; urea = 0 g/L). Phenolic and tannin are expressed as epicatechin equivalents (ECEG). All yields are referred to in kg of dry bark (kg_{DB}).

4.3.3 Successive extractions at increasing temperature

The extraction temperature triggered more intensely the yield of tannins than that of carbohydrates (Figure 31, Figure 32). Consequently, this process parameter was considered a suitable factor to base future investigations on, by developing a simple process able to separate tannins from carbohydrates. Norway spruce and Scots pine bark were thus successively extracted with water at increasing temperatures from 30 to 150°C by multiple steps of 15°C (Bianchi et al., 2016).

For both species, the first extraction steps at 30°C showed a total extraction yield considerably higher than the following step at 45°C. The extraction yield remained almost constant between 45 and 105°C, and then quickly and exponentially increased towards 150°C (Figure 33). In contrast, the phenolic yield, determined by Folin-Ciocalteu assay, remained almost constant throughout all steps (Figure 33). Accordingly, at 30°C and above 105°C the non-phenolic fraction of the extracts was higher than 70%, while in the interval

45-105°C it was between 54% and 65%. Hydrolysis of the bark extracts at 30°C and 150°C showed considerably higher amounts of total carbohydrates than in the extracts at 45°C and 105°C (Table 12). These differences matched with the trend observed for the non-phenolic fractions of the extracts, which were then mostly attributed to carbohydrates.

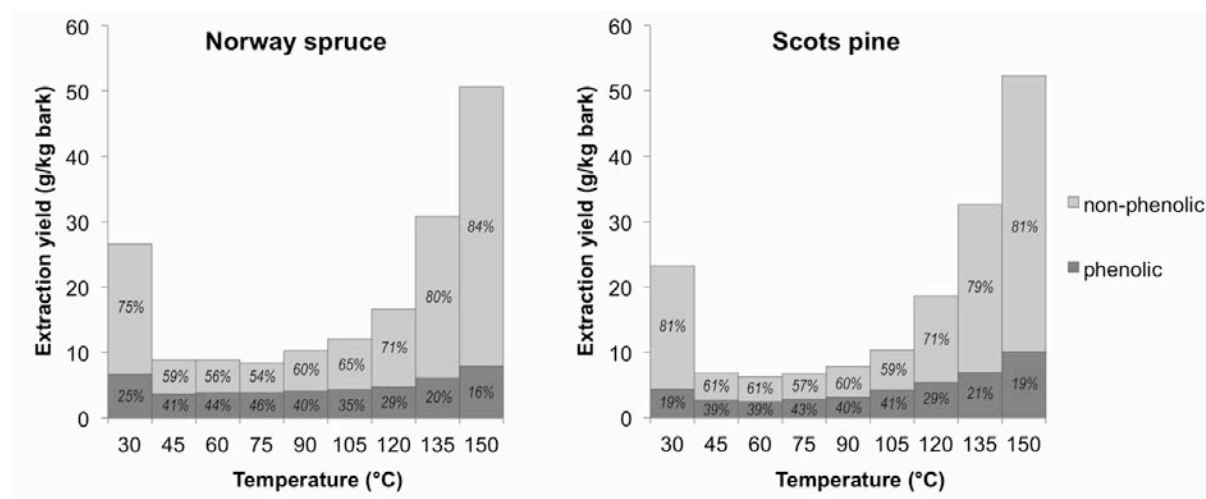


Figure 33: Total and phenolic extraction yield of Norway spruce and Scots pine bark in sequential extractions at increasing temperature. Total phenolic compounds are expressed as purified quebracho tannin equivalents (QEG). The bars report the relative concentration (%) of phenolic and non-phenolic compounds in the extracts (modified from Bianchi et al. 2016).

Changes concerning the composition of phenolics and carbohydrates in the extracts were observed during the successive extractions steps. HPLC-UV chromatograms before thiolysis of the extracts (Figure 34) showed that phenolic monomers were almost completely extracted between 30 and 75°C. Stilbene glucosides almost completely disappeared after 45°C, while taxifolin remained detectable up to 75°C. Accordingly, in the steps above 75°C the extracted phenolic compounds should be mostly represented by oligomers. Thiolysis of Norway spruce extracts showed a progressive decrease of the weight ratio between procyanidins and total phenolics as temperature increased and an abrupt drop in mDP at the 150°C step (Figure 34). Similar changes in the procyanidin structures were observed for Scots pine extracts (data not reported). At increasing extraction temperature a more frequent occurrence of thiolysis resistant oligomers, e.g. phlobaphenes, was thus assumed. These compounds could be either natively developed in the bark or formed from oxidation of procyanidins during the extraction process.

Mono- and oligosaccharides such as glucose, fructose and sucrose were mostly extracted in the first step at 30°C (Table 12). At 45°C and 105°C they were detected only in traces. A higher concentration of arabinose, galactose and rhamnose as free

monosaccharides was observed in the extracts at 150°C, most likely as residues of the thermal degradation of pectins and bark hemicellulose.

Bound carbohydrates in the extracts at 30°C were predominantly represented, in order of decreasing incidence, by glucose, arabinose and galactose residues (Table 12). In the following steps, the relative concentration of glucose residues decreased progressively, likely in relation to the decreasing extraction of stilbene glucosides. At 150°C, higher amounts of arabinose, xylose and mannose residues were detected. The results suggested that pectins like arabinans, arabinogalactans and glucans were extracted throughout the successive extractions. Over 105°C fragments of the bark hemicellulose, such as galactoglucomannans and arabino-4-*O*-methylglucuronoxylans, appeared to be increasingly extracted, as already suggested by Fradinho et al. (2002).

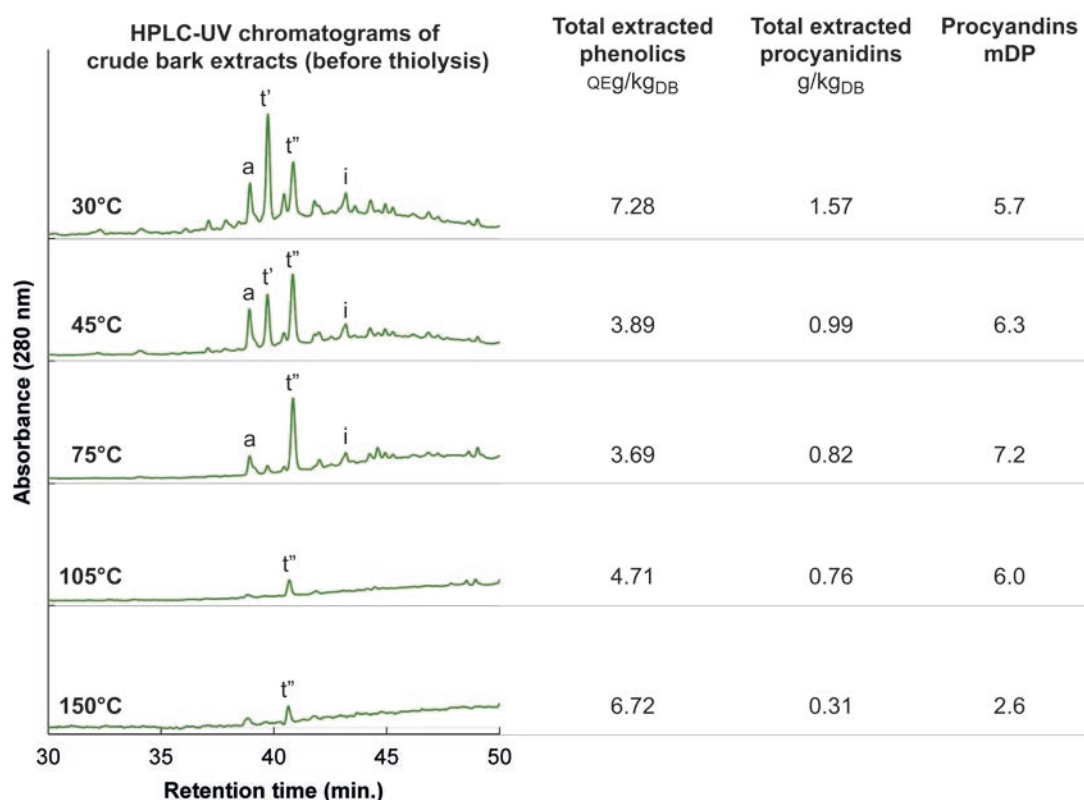


Figure 34: Phenolic composition of Norway spruce bark extracts from successive extractions at increasing temperature. Extracted phenolic monomers are shown in HPLC-UV chromatograms: a = astringin, t'/t'' = taxifolin isomers, i = isorhapontin. Phenolic ratio estimated by Folin-Ciocalteu assay as purified quebracho tannins equivalents (QEG). Total procyanidins amount and their mean degree of polymerization (mDP) estimated by acid thiolysis.

Changes in the composition of Norway spruce extracts during the successive extraction steps were also detected by MALDI-TOF mass spectrometry (Figure 35). In the mass spectra of the extracts at 30°C and 45°C a series of mass peaks attributed to

procyanidins was dominant, showing the presence of oligomers up to 11 units. Low-intensity mass peaks that fitted to pentosans (e.g. arabinans) were also detected. At 105°C the mass spectra considerably changed: the peak series associated to procyanidins was less intense, while the pentosan series became more important and overlaid with that of procyanidins. At 150°C the procyanidins' peaks could be barely recognized and the pentosan series was clearly dominant. The progressive disappearance of procyanidins and the increasing concentration of polysaccharides during successive extractions at increasing temperature were then confirmed.

Table 12: Composition of total, free and bound carbohydrates in Norway spruce and Scots pine bark extracts by successive extractions at increasing temperatures (n = 1).

Free carbohydrates are associated to mono- and oligosaccharides

Bound carbohydrates are associated to polysaccharides and glycosyl residuals

Extraction step	Carbohydrates (g/kg _{DB})											
	Glc	Gal	Fru	Xyl	Man	Ara	Rha	Suc	Raf	TOTAL		
Norway spruce	30 °C	Total	3.79	1.40	0.50	0.35	0.29	1.64	0.32	-	-	8.29
		Free	0.56	<0.01	0.59	<0.01	<0.01	0.20	0.02	0.53	0.07	2.60
		Bound	2.93	1.38	<0.01	0.35	0.29	1.44	0.30	-	-	6.69
	45 °C	Total	0.86	0.42	<0.01	0.11	0.12	0.45	0.07	-	-	2.02
		Free	0.05	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	0.08
		Bound	0.81	0.42	<0.01	0.11	0.12	0.45	0.06	-	-	1.96
	105 °C	Total	1.00	0.69	<0.01	0.18	0.29	1.39	0.10	-	-	3.66
		Free	0.01	<0.01	<0.01	<0.01	<0.01	0.22	0.01	<0.01	<0.01	0.25
		Bound	0.99	0.69	<0.01	0.18	0.29	1.17	0.09	-	-	3.41
	150 °C	Total	2.65	4.85	<0.01	2.56	2.07	9.37	0.73	-	-	22.24
		Free	0.12	0.30	<0.01	0.14	<0.01	2.40	0.20	<0.01	<0.01	3.16
		Bound	2.53	4.55	<0.01	2.42	2.07	6.98	0.53	-	-	19.08
Scots pine	30 °C	Total	3.57	1.15	0.71	0.36	0.37	2.56	0.43	-	-	9.14
		Free	0.35	0.03	0.61	0.01	<0.01	0.33	0.02	0.13	0.03	1.72
		Bound	3.13	1.12	0.01	0.34	0.37	2.23	0.40	-	-	7.61
	45 °C	Total	0.61	0.53	<0.01	0.18	0.20	1.11	0.12	-	-	2.74
		Free	0.04	0.01	0.01	<0.01	<0.01	0.01	0.01	0.01	<0.01	<0.09
		Bound	0.57	0.51	<0.01	0.18	0.20	1.11	0.11	-	-	2.67
	105 °C	Total	0.81	0.55	<0.01	0.21	0.11	2.27	0.23	-	-	4.18
		Free	0.01	0.02	<0.10	0.01	0.02	0.36	<0.01	<0.01	<0.01	0.42
		Bound	0.80	0.53	<0.01	0.20	0.09	1.90	0.23	-	-	3.75
	150 °C	Total	3.32	6.74	<0.01	4.00	2.89	8.91	0.93	-	-	26.79
		Free	0.28	1.00	<0.01	0.52	<0.01	4.18	0.36	<0.01	<0.01	6.34
		Bound	3.04	5.75	<0.01	3.48	2.89	4.72	0.57	-	-	20.45

kg_{DB} = kg of dry bark

The analysis of Norway spruce bark extracts during successive extractions highlighted the existence of three extraction temperature ranges. The first appeared in the initial extraction step ($\leq 45^\circ\text{C}$), where native mono-/oligosaccharides, a large fraction of the phenolic monomers and part of the tannins were extracted. The second temperature range appeared between 45 and 105°C . Within these temperatures the extracts were much richer in procyanidins, and pectins like arabinans, arabinogalactans and glucans mainly represented the non-phenolic fraction. The third range was above 105°C , where pectins represented the majority of the extract and the extracted phenolics were most likely highly oxidized oligomers (e.g. phlobaphenes). The composition of the extracts in these three ranges roughly resembled those obtain by ultrafiltration of the Radiata pine extracts through molecular membranes of 10^3 and 10^6 Da in size (Yazaki and Hillis 1985).

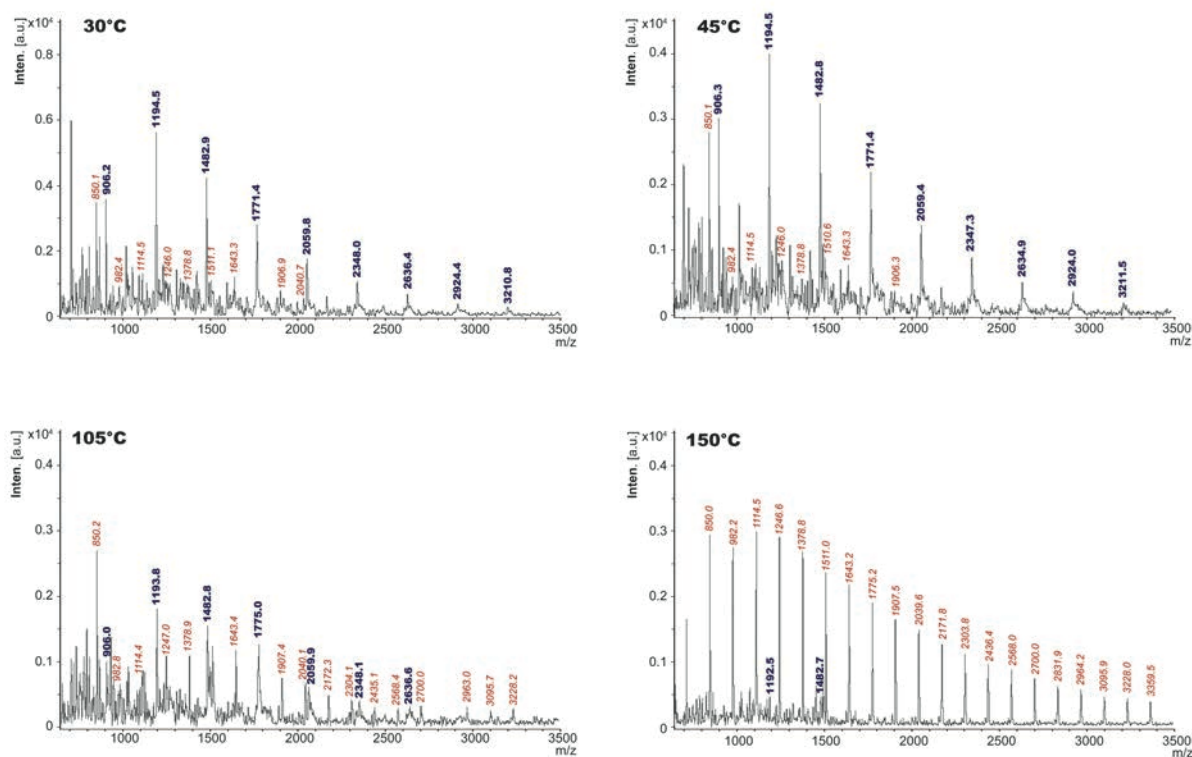


Figure 35: MALDI-TOF mass spectra of Norway spruce bark extracts obtained from successive extractions at increasing temperature. In each spectrum, a peak series associated to procyanidins (**blue bold**) and pentosans (*red italic*) are labelled.

4.3.4 Prolonged storage of bark exposed to natural weathering

A 15 m long Norway spruce log was left exposed for 469 days (approximately 15 months) to natural weathering starting from the day of tree felling. Samples were collected at 1 m and 10 m from the stump and different storage times and the variations in the

phenolic deposits and total tannins within the bark were investigated by UMSP and direct thiolytic, respectively (Bianchi et al., 2016). Between 1 and 210 days, A_{280} of bark samples collected at 10 m slightly decreased from 0.609 to 0.565. Similarly, in the samples collected at 1 m a slight decrease of A_{280} from 0.649 to 0.612 was observed. The higher A_{280} in correspondence of the stump suggested a higher lignification of these samples, roughly associated to older bark tissues. However, point UV spectra of both bark cell walls and phenolic deposits didn't significantly change along the log and during storage. Direct thiolytic of fine milled bark collected at 1, 210 and 469 days indicated a decreasing trend in total procyanidins (Table 13). The leaching and/or degradation of the bark procyanidins during the log storage were therefore expected.

Table 13: Extracted phenolics and total, extracted and non-extractable procyanidins of Norway spruce bark samples after different durations of storage (Mean \pm Std.Dev; n = 3)

Position	Storage time	Phenolic compounds			Procyanidins				
		Total	Monomers (F1)	Oligomers (F2)	Total	Extracted		Non-extractable	
		ECEg/kg _{DB}	ECEg/kg _{DB}	QEg/kg _{DB}	g/kg _{DB}	mDP	g/kg _{DB}	mDP	g/kg _{DB}
	1 day	22.1 \pm 1.1	10.3 \pm 0.6	15.7 \pm 0.7	22.8 \pm 1.2	7.2 \pm 1.1	8.6 \pm 0.9	6.3 \pm 0.9	14.2 \pm 1.5
1 m	210 days	10.1 \pm 0.4	3.3 \pm 0.3	8.6 \pm 0.4	17.5 \pm 0.9	7.7 \pm 1.2	2.8 \pm 0.3	4.7 \pm 0.7	14.6 \pm 0.9
	469 days	6.5 \pm 0.3	1.8 \pm 0.2	5.6 \pm 0.1	14.4 \pm 0.7	7.7 \pm 1.2	1.3 \pm 0.3	4.8 \pm 0.7	13.0 \pm 0.8
	1 day	31.5 \pm 1.5	15.3 \pm 0.9	22.4 \pm 1.1	21.6 \pm 1.0	6.3 \pm 0.9	9.9 \pm 0.9	5.5 \pm 0.8	11.0 \pm 1.0
10 m	210 days	13.3 \pm 0.7	4.5 \pm 0.3	10.8 \pm 0.4	15.2 \pm 0.7	6.7 \pm 1.0	2.8 \pm 0.3	4.4 \pm 0.7	12.4 \pm 0.8
	469 days	8.4 \pm 0.3	2.7 \pm 0.2	7.4 \pm 0.4	14.4 \pm 0.8	7.3 \pm 1.1	1.7 \pm 0.2	3.8 \pm 0.6	12.7 \pm 0.8

mDP = mean degree of polymerization
 ECEg = g of (-)-epicatechin equivalents
 QEg = g of purified quebracho tannin equivalents
 kg_{DB} = kg of dry bark

The bark samples collected during the storage of the log were extracted in water at 60°C and the extracts characterized in regarding the phenolic, tannin and carbohydrate contents (Bianchi et al., 2016). Samples collected at 10 m consistently showed a higher total extraction yield than samples collected at 1 m, in agreement with König and Roffael (2002). For both sampling positions, a progressive decrease in the total extraction yield was observed along the log storage (Figure 36). The phenolic yield decreased proportionally to the total yield, accordingly to the constant concentration of total phenolics in the extracts both along the log height and during the storage (Figure 36). A slight decreasing trend in the phenolic concentration was observed only after 200 days (Figure 36).

The change in the composition of the extracted phenolics over the storage time period was observed. Extracts from fresh bark (from day 1) had a very comparable share of phenolic monomers and oligomers. In samples collected at 210 days, preponderance of phenolic oligomers was instead detected (Table 13). HPLC-UV chromatograms of the extracts

from bark collected at 10 m confirmed these results, showing an evident reduction of the peaks associated to astringin, taxifolin and isorhapontin during storage time (Figure 37). The intensities of the phenolic monomer peaks for bark samples collected at 1 m (data not reported) were significantly lower than those observed at 10 m, in agreement with the differences in the phenolic monomer yields (fraction F1) observed between the extracts at the two positions (Table 13). A decrease of the concentration of phenolic monomer during *in vivo* aging of the bark was therefore supposed.

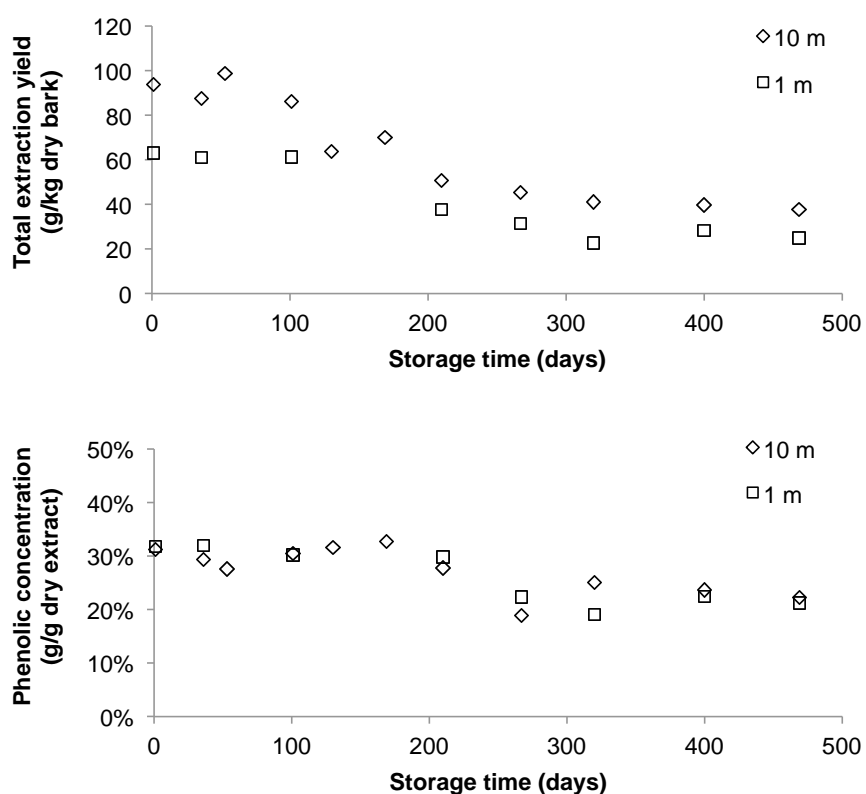


Figure 36: Total extraction yield and concentration of phenolic compounds in the extracts during outdoor storage of Norway spruce bark (samples collected at 1 and 10 m from the stump) (modified from Bianchi et al. 2016).

Changes in the structure of the extracted condensed tannins during bark storage were observed by MALDI-TOF mass spectrometry. In the sample collected at 1 day after tree felling, a mass peak series associated to pure procyanidins dominated the mass spectrum of the extracts (Figure 38). In the same mass spectrum, peak series relative to procyanidins containing one or two astringin units were observed at a considerably lower intensity. The lower concentration of such oligomers in the extracts was thus suggested. In the MALDI-TOF mass spectrum of extracts at 210 days (Figure 38), the intensity of the pure procyanidin series was almost comparable to the oligomer series containing astringin units. Thus, an

almost equal occurrence of both tannin types in the extracts at this storage time was indicated. At 469 days few and very low intense tannin peaks were detected in the mass spectrum, even if the phenolic fraction should be almost completely represented by oligomers (Table 13 and Figure 38). The extraction of oligomers with a less ordered structure than condensed tannins was therefore considered. The progressive decrease of the polymerization degree of condensed tannins is similar to what observed in Norway spruce needle litter (Maie et al. 2003).

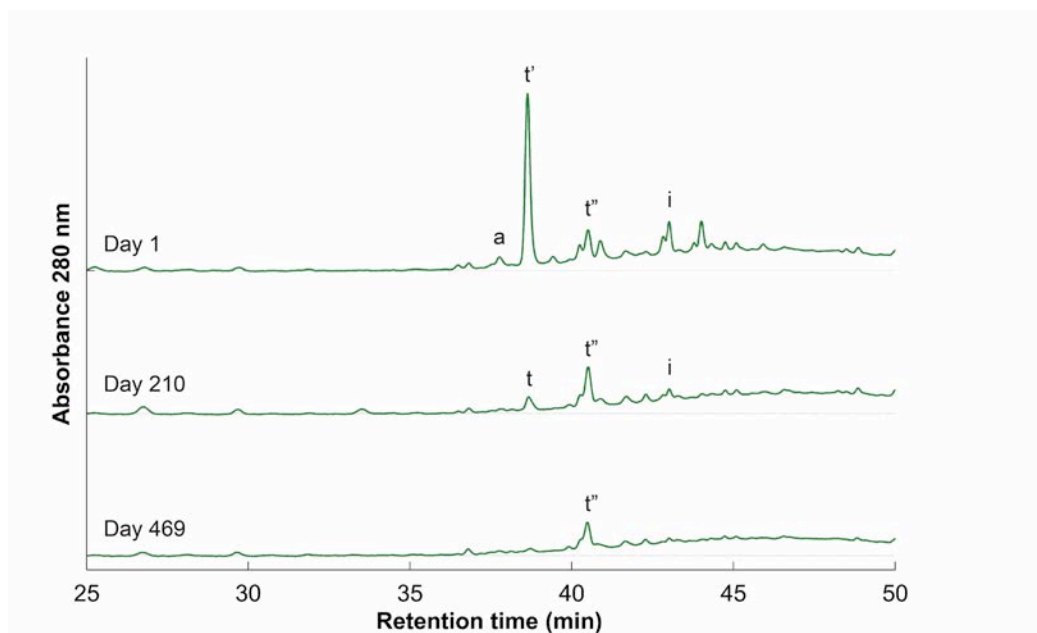


Figure 37: HPLC-UV chromatograms of Norway spruce extracts from samples collected at different bark weathering time. The absorption peaks were associate to: a = astringin, t'/t'' = taxifolin isomers, i = isorhapontin.

The carbohydrate composition also varied along storage. Mono- and oligosaccharides, mainly represented by glucose, fructose and sucrose, were detected in relevant amounts only in fresh bark extract (1 day; Table 14). Only traces of glucose were identified in the extracts at 210 days. Bound carbohydrates (polysaccharides and glycosyl residues) diminished as well, but remained detectable in a considerable amount in the more aged samples (Table 14). The major contribution to their reduction was the rapid decrease of glucose residues, which could be partially caused by the decrease in the extracted stilbene glucosides observed by HPLC-UV (Figure 37). The sum of carbohydrate residues other than glucose (mostly arabinose, galactose and galaturonic acid) was more stable along, both, the log position and during storage (5.5 – 11.3 g/kg_{D_B}). These residues were associated to pectins, which showed thus a extraction availability during bark aging more constant than other extractives.

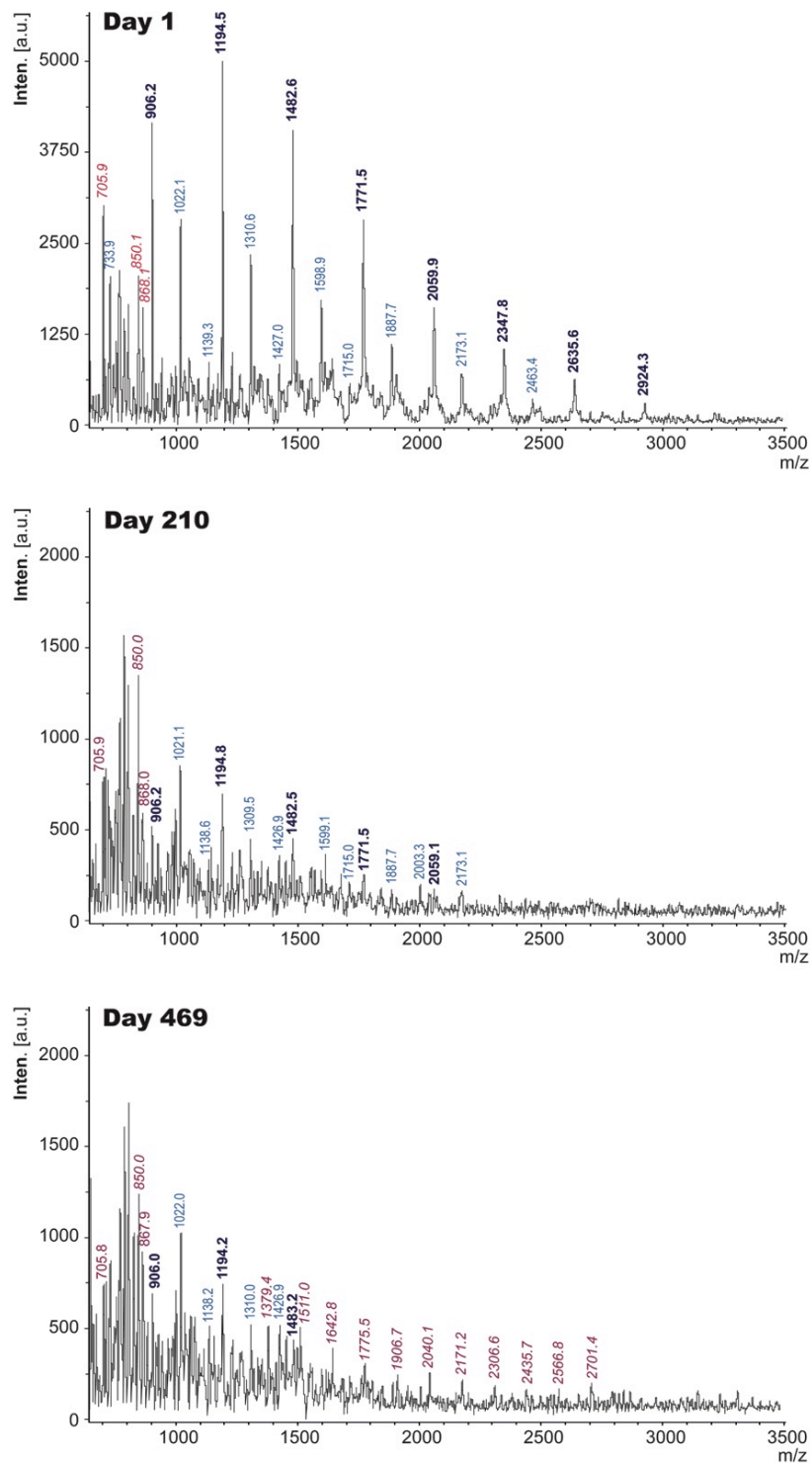


Figure 38: MALDI-TOF mass spectra of Norway spruce bark extracts from samples collected at 1 and 210 days of outdoor storage. In each spectrum the following polymer series were identified: pure procyanidins (**blue bold**), procyanidins substituted with astringin units (**blue narrow**), oligo-/polysaccharides (*red italic*).

Table 14: Composition of total, free and bound carbohydrates in Norway spruce extracts from samples collected at different bark weathering time. (n = 1)

Free carbohydrates are associated to mono- and oligosaccharides

Bound carbohydrates are associated to polysaccharides and glycosyl residues

		Carbohydrates (g/kg _{DB})											
		Glc	Gal	Fru	Xyl	Man	Ara	Rha	GalA	Suc	Raf	TOTAL	
Position = 1 m	1 day	Total	10.80	1.90	1.86	0.50	0.45	3.19	0.41	1.17	-	-	20.35
		Free	1.22	0.03	1.72	<0.01	<0.01	0.05	<0.02	<0.01	2.13	0.10	5.40
		Bound	8.41	1.83	<0.01	0.50	0.45	3.14	0.39	1.17	-	-	15.98
	210 days	Total	4.60	1.22	0.04	0.35	0.39	2.27	0.44	0.87	-	-	10.28
		Free	0.46	0.02	0.02	<0.01	<0.01	0.09	<0.01	0.07	0.03	0.02	0.22
		Bound	4.13	1.21	0.03	0.35	0.39	2.18	0.44	0.79	-	-	9.59
Position = 10 m	1 day	Total	17.01	2.79	2.15	0.66	0.67	4.87	0.72	1.80	-	-	30.77
		Free	1.65	0.04	1.90	<0.01	<0.01	0.03	0.03	0.13	2.85	0.14	6.91
		Bound	13.82	2.71	<0.01	0.66	0.67	4.84	0.69	1.68	-	-	25.16
	210 days	Total	6.51	1.91	0.06	0.56	0.87	3.15	0.60	1.29	-	-	15.07
		Free	0.46	0.01	0.12	<0.01	<0.01	0.07	<0.01	<0.01	0.07	0.01	0.74
		Bound	6.01	1.90	<0.01	0.56	0.87	3.07	0.60	1.29	-	-	14.43

kg_{DB} = kg of dry bark

The prolonged storage of Norway spruce bark exposed to natural weathering was thus accompanied by substantial changes in extraction yield and extract composition, which has to be taken into account for the industrial exploitation of softwood barks. Compounds that could be extracted in cold water, like mono-/oligosaccharides, phenolic monomers and a fraction of the extractable tannins (as discussed in the section 4.3.3), were progressively missing in the extracts during bark storage, causing a consequent decrease in the total extraction yield. Fresh bark samples that were freeze-dried and then stored at room temperature in dry and dark conditions showed a negligible reduction of the total and phenolic extraction yield over 469 days (Bianchi et al. 2016), suggesting that, in the absence of weathering and moisture, no substantial changes in the bark occurred. The depletion of the most extractable compounds was thus mainly attributed to leaching, biological consumption or degradation. A reduction in the extraction yield associated to the development of secondary bonds among phenolic oligomers during aging (Matthews et al. 1997b) seemed to represent a negligible factor. Pectins and highly condensed phenolic oligomers (e.g. phlobatannin, phlobaphenes) were constantly present in the bark extracts and represented their major components at the longest storage times.

Similar outcomes resulted when a comparison between bark tissues of different ages was made. A lower recovery in the most extractable compounds was observed close to the stump (older tissue) than 10 m from it (younger tissue). However, in this case, older tissues

had a slightly higher amount of non-extractable procyanidins than younger tissues (Table 13), suggesting that, during *in vivo* aging, secondary bonds between phenolic extractives are likely developed.

5. Conclusions

The possibility of recovering condensed tannins from the bark of European softwood species through hot water extractions was investigated. The barks of Silver fir, European larch, Norway spruce, Douglas fir and Scots pine, five of the most common softwoods in North and Central Europe, were extracted at 60°C and the chemical composition of the extracts analyzed. The use of a consistent method in both the extraction and the chemical characterization enabled identifying differences and similarities among the investigated softwood species without uncertainties related to dissimilar analytical procedures.

The total yield of extraction was between 26.9 and 120.2 g/kg_{DB}, with the lowest value corresponding to Scots pine and the highest to Silver fir. These figures were considerably low when compared to the total extractives in softwood barks (estimated by organic solvent extraction), which was three times higher, and the extraction yields of currently used tannin-rich extracts from tropical species, which are generally higher than 200 g/kg_{DB}. A further weakness concerned the composition of the softwood bark extracts. Phenolic compounds represented only between 13% (Scots pine) and 46% (European larch) of the extracts, and a comparable amount of carbohydrates (21%-45%) was concurrently extracted.

Phenolic compounds were represented by both monomers (e.g. stilbene glucosides, taxifolin) and oligomers and, among these, condensed tannins. The improvement of existing protocols for MALDI-TOF mass spectrometry and thiolysis followed by HPLC-UV provided deep insights into the extracted condensed tannins' molecular structure. In contrast to the tannins from tropical species, which were described as mostly made of 5-dehydroxylated flavan-3-ol trimers and tetramers, European softwood tannins showed more complex structures. Along the procyanidin/prodelphinidin backbone, substitution with stilbene glucosides, glucosylation, galloyl-esters, and most likely side-branches were identified. Their mean degree of polymerization was between 3.5 (Silver fir) and 6.7 (Scots pine), and the presence of oligomers up to 13 units was observed. The recurrent occurrence of oxidized configurations and rearrangements (e.g. phlobatannins) was hinted.

These findings advised that condensed tannins extracted from European softwood bark cannot be regarded as simple linear procyanidins or prodelphinidins as they are commonly reported in the literature. Their convoluted molecular structure might result in the hindrance of reactive sites and in the reduction of the already low flexibility of tannin oligomers. This will consequentially influence the chemical and physical properties of the bark extracts. Accordingly, the technologies and resin formulations developed for tropical

tannins cannot simply be superimposed onto softwood bark extracts. Most likely, alternative solutions, e.g. new resin cross-linkers, have to be developed. Differences among the molecular structures of tannins extracted from different softwood species also suggested distinct properties among them, and the need of specific solutions for the extracts of each species.

Glucose, fructose and pectins were the main carbohydrates detected in extracts. In particular, pectins, which were mainly represented by arabinans, arabinogalactans and glucans, showed a quite homogenous yield across the species and during outdoor storage of the bark. Wide variations were instead observed in the monosaccharide and phenolic monomer yields. These variations were not only associated to native characteristics of the softwood species, but also to the storage history of the bark. The concentration of small molecular weight extractives constantly decreased in bark samples from a log left exposed to natural weathering, most likely because of leaching, degradation or biological consumption. The most extractable tannins also diminished during bark storage. Accordingly, relevant changes in the extraction yield and the composition of the extracts were detected during bark aging: an aspect seldom considered, but critical in view of the industrial exploitation of bark extracts.

The temperature was the most influencing factor on the bark extraction process. Successive extractions at increasing temperature enabled partial separation of the components of bark extracts. Monosaccharides and phenolic monomers, the main components responsible for the variations in yield and extract composition during bark aging, could be almost completely extracted at temperatures below or equal to 45°C. The process of successive extractions in the range 45 – 105°C was able to provide extracts richer in phenolic oligomers and therefore more suitable to be used as phenolic substitutes in resin formulations. However, the sizable amount of tannins already extracted in the first steps at lower temperature, reduced the net yield of the phenolic-rich extracts. This aspect should be carefully considered in the evaluation of the economic feasibility. An identification of valuable applications for both of the extracts obtained at different temperatures will benefit the overall economy of the process. Extraction at temperatures over 100°C were discouraged, being characterized by severe tannin oxidation and/or rearrangement, and a dramatic increase in the extraction of pectins. Addition of sulfites and/or urea was likewise considered futile, as both didn't provide any actual improvement in phenolic yield or phenolic carbohydrate ratio. The concurrent extraction of tannins and pectins couldn't be avoided in any process conditions.

To increase the purity of water-extracted tannins a post-processing of the extracts is needed. Possible options are represented by pectin precipitation by addition of alcohols, ketones or calcium ions, ultrafiltration, or *in situ* enzymatic/biological degradation of the polysaccharides. The implementation of such processes will strongly impact the production

cost of the extracts, which will become less attractive, in particular for low-value applications, e.g. tannin-based wood adhesives. Direct extraction of the phenolics from the bark using organic solvents (e.g. ethanol, acetone) might be a cheaper alternative. The necessary grade of tannin purification should be carefully evaluated in relation to the final application of the extracts. A clear understanding of the influence of non-tannin compounds on the properties of the bark extracts, besides the simple dilution effect, has not yet been developed. Specific investigations should be considered on a case-by-case basis. Alternative solutions are worth investigating. The coexistence of carbohydrates and phenolic oligomers could be regarded as a distinctive characteristic of softwood bark extracts worth exploiting. Possible co-reactions and synergies between these compound classes should be further investigated.

Non-extractable and non-leachable phenolic deposits in the lumen of bark cells were detected. Condensed tannins with a molecular structure similar to those extracted were detected among these non-extractable compounds. These tannins could be regarded as immobilized oligomers that likely maintained their chemical reactivity (e.g. condensation with aldehydes, heavy-metal chelation ability). The extracted bark represents therefore a substrate still rich in tannin-like compounds that is worth valorizing. Investigation of possible uses of extracted bark (e.g. formaldehyde scavenger, wastewater flocculation, etc.) should be considered.

6. Literature

6.1 Author's publications

- Bianchi, S., Glöss, A.N., Krosiakova, I., Mayer, I., Pichelin, F. (2014). Analysis of the structure of condensed tannins in water extracts from bark tissues of Norway spruce (*Picea abies* [Karst.]) and Silver fir (*Abies alba* [Mill.]) using MALDI-TOF mass spectrometry. *Ind. Crop. Prod.* 61: 430-437.
- Bianchi, S., Krosiakova, I., Janzon, R., Mayer, I., Saake, B., Pichelin, F. (2015). Characterization of condensed tannins and carbohydrates in hot water bark extracts of European softwood species. *Phytochemistry* 120: 53-61.
- Bianchi, S., Koch, G., Janzon, R., Mayer, I., Saake, B., Pichelin, F. (2016). Hot water extraction of Norway spruce (*Picea abies* [Karst.]) bark: analyses of the influence of bark aging and process parameters on the extract composition. *Holzforschung* 70: 619-631.

6.2 Other publications

- Altaner C.M., Tokareva, E.N., Jarvis, M.C., Harris, P.J. (2010). Distribution of (1 →4) -β-galactans, arabinogalactan proteins, xylans and (1 →3) -β-glucans in tracheid cell walls of softwoods. *Tree Physiol.* 30: 782-793.
- Appel, H.M., Govenor, H.L., D'Ascenzo, M., Siska, E., Schultz, J.C. (2001). Limitations of Folin assays of foliar phenolics in ecological studies. *J. Chem. Ecol.* 27: 761-778.
- Aspé, E., Fernández, K. (2011). The effect of different extraction techniques on extraction yield, total phenolic, and anti-radical capacity of extracts from *Pinus radiata* bark. *Ind. Crop. Prod.* 34: 838-844.
- Bae, Y.S., Malan, J.C.S., Karchesy, J.J. (1994). Sulfonation of procyanidin polymers: evidence of intramolecular rearrangement and aromatic ring substitution. *Holzforschung* 48: 119-123.
- Ballerini, A., Depres, A., Pizzi, A. (2005). Non-toxic, zero emission tannin-glyoxal adhesives for wood panels. *Holz Roh. Werkst.* 63: 477-478.
- Barbosa, V., Ramires, E.C., Razera, I.A.T, Frollini, E. (2010). Biobased composites from tannin-phenolic polymers reinforced with coir fibers. *Ind. Crop. Prod.* 32: 305-312.
- Bartolomé, B., Bengoechea, M.L., Gálvez, M.C., Pérez-Illzarbe, F.J., Hernández, T., Estrella, I., Gómez-Cordovés, C. (1993). Photodiode array detection for elucidation of the structure of phenolic compounds. *J. Chromatog. A* 655: 119-125.
- Barton, G.M., Gardner, J.A.F., (1958). Determination of Dihydroquercetin in Douglas fir and Western Larch Wood. *Anal. Chem.* 30: 279-281.
- Benkovic, E.T., Grohar, T., Zigon, D., Svajger, U., Janes, D., Kreft, S., Strukelj, B. (2014). Chemical composition of the silver fir (*Abies alba*) bark extract Abigenol® and its antioxidant activity. *Ind. Crop. Prod.* 52: 23-28.
- Bertaud, F., Tapin-Lingua, S., Pizzi, A., Navarrete, P., Petit-Conil, M. (2012). Development of Green Adhesives for Fiberboard Manufacturing using Tannins and Lignin from Pulp Mill Residues. *Cell. Chem. Technol.* 46: 449-455.

- Botha, J.J., Ferreira, D., Roux, D.G. (1978) Condensed tannins - Direct synthesis, structure and absolute-configuration of 4 biflavonoids from Black wattle bark (*Mimosa*) extract. *J. Chem. Soc., Chem. Comm.*: 700-702.
- Burger, J.F.W., Kolodziej, H., Hemigway, R.W., Steynberg, J.P., Young, D.A., Ferreira, D. (1990). Oligomeric flavonoids. Part 15. Base-catalyzed pyran rearrangement of procyanidin B-2, and evidence for the oxidative transformation of B- to A-type procyanidins. *Tetrahedron* 46: 5733-5740.
- Chen, C.M., Pan, J.K. (1991). Effects of extraction on yields and characteristics of bark extracts. *Holzforschung* 45: 155-159.
- Chupin, L., Motillon, C., Charrier-El Bouhtoury, F., Pizzi, A., Charrier, B. (2013). Characterization of maritime pine (*Pinus pinaster*) bark tannins extracted under different conditions by spectroscopic methods, FTIR and HPLC. *Ind. Crops Prod.* 49: 897-903.
- Clifford, M.N., Stoupi, S., Kuhnert, N. (2007). Profiling and characterization by LC-MSn of the galloylquinic acids of green tea, tara tannin and tannic acid. *J. Agric. Food Chem.* 55: 2797-2807.
- Čop, M., Lacoste, C., Conradi, M., Laborie, M.P., Pizzi, A., Sernek, M. (2015). The effect of the composition of spruce and pine tannin-based foam on their physical, morphological and compression properties. *Ind. Crop. Prod.* 74: 158-164.
- De Bruyne, T., Pieters, L., Deelstra, H., Vlietinck, A. (1999). Condensed vegetable tannins: biodiversity in structure and biological activities. *Biochem. Syst. Ecol.* 27: 445-459.
- Dietrich, H.H., Garves, K., Behrendorf, D., Sinner, M. (1978). Untersuchung über die Kohlenhydrate der Rinden einheimischer Holzarten. *Holzforschung* 32: 60-67.
- Diouf, P.N., Stevanovic, T., Cloutier, A. (2009). Study on chemical composition, antioxidant and anti-inflammatory activities of hot water extracts from *Picea mariana* bark and its proanthocyanidin-rich fraction. *Food Chem.* 113: 897-902.
- Dix, B., Marutzky, R. (1983). Untersuchungen zur Gewinnung von Polyphenolen aus Nadelholzrinden. *Holz Roh. Werkst.* 41: 45-50.
- Dix, B., Marutzky, R. (1987). Tanninformaldehydharze aus den Rindenextrakten von Fichte (*Picea abies*) und Kiefer (*Pinus sylvestris*). *Holz Roh. Werkst.* 45: 457-463.
- EUROSTAT (2015). Agriculture forestry and fishery statistics. 2014 Edition. Publisher: European Union, pp. 141-165.
- Everette J.D., Bryant, Q.M., Green, A.M., Abbey, Y.A., Wangila, G.W., Walker, R.B. (2010). Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent. *J. Agr. Food Chem.* 58: 8139-8144.
- Evert, R.Y. (2006). Cell Wall. In: Esau's plant anatomy, Evert, R.Y. Ed.; John Wiley and Sons Inc., New Jersey; pp. 65-101.
- Feng, S., Cheng, S., Yuan, Z., Leitch, M., Xu, C. (2013). Valorization of bark for chemicals and materials: a review. *Renew. Sust. Ener. Rev.* 26: 560-578.
- Fengel, D., Wegener, G. (1983a). Polyoses (Hemicellulose). in: *Wood Chemistry, Ultrastructure, Reactions*. Walter de Gruyter, Berlin, 106-131.
- Fengel, D., Wegener, G. (1983b). Bark Constituents. in: *Wood Chemistry, Ultrastructure, Reactions*. Walter de Gruyter, Berlin, 240-267.
- Ferreira, D., Steynberg, J.P., Burger, J.F.W., Bezuidenhout, C.B. (1992). Oxidation and rearrangement reactions of condensed tannins. In: Hemingway, R.W., Laks, P.E. (Eds.), *Plant Polyphenols*, Plenum Press, New York, pp. 349-384.
- Field, J.A., Lettinga, G. (1992). Biodegradation of tannins. In: *Metal ions in biological systems Vol. 28*. Sigel, H. and Sigel, A. (Eds). Marcel Dekker, New York, p. 61-97.

- Foo, L.Y., McGraw, G.W., Hemingway, R.W. (1983). Condensed tannins: preferential substitution at the interflavonoid bond by sulphite ion. *J. Chem. Soc., Chem. Comm.*: 672-673.
- Foo, L.Y., Karchesy, J.J. (1989a). Chemical nature of phlobaphenes. In: *Chemistry and significance of condensed tannins*, Hemingway, R.W., Karchesy, J.J. Eds.; Publisher: Plenum Press, New York; pp. 109-118.
- Foo, L.Y., Karchesy, J.J., (1989b). Polyphenolic glycosides from Douglas fir inner bark. *Phytochemistry*, 28: 1237-1240.
- Foo, L.Y., Karchesy, J.J., (1989c). Procyanidins dimers and trimers from Douglas fir inner bark. *Phytochemistry*, 28: 1743-1747.
- Foo, L.Y., Karchesy, J.J., (1989d). Procyanidin polymers of Douglas fir bark: structure from degradation with phloroglucinol. *Phytochemistry*, 28: 3185-3190
- Foo, L.Y., Karchesy, J.J., (1991). Procyanidins tetramers and pentamers from Douglas fir bark. *Phytochemistry*, 30: 667-670.
- Foo, L.Y., Porter, L.J. (1980). The phytochemistry of proanthocyanidin polymers. *Phytochemistry* 19: 1747-1754.
- Fradinho, D.M., Pascoal Neto, C., Evtuguin, D., Jorge, F.C., Irle, M.A., Gil, M.H., Pedrosa de Jesus, J. (2002). Chemical characterisation of bark and of alkaline bark extracts from maritime pine grown in Portugal. *Ind. Crop. Prod.* 16: 23-32.
- Fu, Y.L., Gutmann, P.J., Timell, T.E. (1972). Polysaccharides in the secondary phloem of Scots pine (*Pinus sylvestris* L.). I. Isolation and characterization of callose. *Cell. Chem. Technol.* 6: 507-512.
- Fu, Y.L., Timell, T.E. (1972a). Polysaccharides in the secondary phloem of Scots pine (*Pinus sylvestris* L.). II. The structure of an arabinan. *Cell. Chem. Technol.* 6: 513-515.
- Fu, Y.L., Timell, T.E. (1972b). Polysaccharides in the secondary phloem of Scots pine (*Pinus sylvestris* L.). III. The constitution of a galactoglucomannan. *Cell. Chem. Technol.* 6: 517-519.
- Gaballah, I., Kilbertus, G. (1998). Recovery of heavy metal ions through decontamination of synthetic solutions and industrial effluents using modified barks. *J. Geochem. Explor.* 62: 241-286.
- Ganjegunte, G.K., Condrón, L.M., Clinton, P.W., Davis, M.R., Mahieu, N. (2004). Decomposition and nutrient release from radiata pine (*Pinus radiata*) coarse woody debris. *Forest Ecol. Manag.* 187: 197-211.
- Garnier, S., Pizzi, A., Vorster, O.C., Halasz, L. (2001). Comparative rheological characteristics of industrial polyflavonoid tannin extracts. *J. Appl. Polym.* 81: 1634-1642.
- Goldschmid, O. (1975). Ultraviolet spectra. In: *Lignins, Occurrence, Formation, Structure and Reactions*. Sarkanen, K.V., Ludwig, C.H. Eds.; Publisher: Wiley Interscience, New York; pp. 241-266.
- Hagerman, A.E., Butler, L.G. (1989). Choosing appropriate methods and standards for assaying tannin. *J. Chem. Ecol.* 15: 1795-1810.
- Hagerman, A.E., Riedl, K.M., Rice, R.E. (1999). Tannins as biological antioxidants. In: *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*. Eds: Gross et al. Kluwer Academic / Plenum Publishers, New York; pp. 495-505.
- Hara, Y., Mizukawa, H., Yamamoto, H., Ikami, T., Kato, K., Yabe, T. (2013). Simple method for refining arabinan polysaccharides by alcohol extraction of the Prune, *Prunus domestica* L. *Biosci. Biotechnol. Biochem.* 77: 2137-2139.
- Harborne, J.B. (1977). Flavonoids and the evolution of angiosperms. *Biochem. System. Ecol.* 5: 7-22.
- Hashida, K., Ohara, S., Makino, R. (2006). Base-catalyzed reactions of procyanidin B3: formation of a novel catechinic acid-catechin dimer. *J. Wood Chem. Technol.* 26: 125-140.
- Haslam, E. (1989a). Polyphenols – Vegetable tannins. in: *Plant polyphenols: vegetable tannins revisited*; University Press, Cambridge, UK; pp. 1-13.

- Haslam, E. (1989b). Gallic acid derivatives and hydrolysable tannins. in: *Natural Products of Woody Plants*; Rowe, J.W. Ed., Springer-Verlag, Berlin, Germany; vol. 1, pp. 399-438.
- Heinrich, H., Pichelin, F., Pizzi, A. (1996). Lower temperature tannin/hexamine-bonded particleboard of improved performance. *Holz Roh. Werkst.* 54: 262.
- Hellström, J.K., Mattila, P.H. (2008). HPLC determination of extractable and unextractable proanthocyanidins in plant materials. *J. Agr. Food Chem.* 56: 7617-7624.
- Hemingway, R.W. (1989). Structural variations in proanthocyanidins and their derivatives. In: Hemingway, R.W., Karchesy, J.J. (Eds.), *Chemistry and Significance of Condensed Tannins*. Plenum Press, New York, pp. 83-107.
- Hemingway, R.W., McGraw, G.W. (1983). Kinetics of acid-catalyzed cleavage of procyanidins. *J. Wood Chem. Technol.* 3: 421-435.
- Hergert, H.L. (1992). The nature of non-proanthocyanidin units in 'condensed tannins' from conifer wood and bark. In: Hemingway, R.W., Laks, P.E. (Eds.), *Plant Polyphenols, Basic Life Sciences*, vol. 59. Springer Science + Business Media, New York, pp. 385-409.
- Hillis, W.E. (1985). Occurrence of extractives in wood tissues. In: *Biosynthesis and biodegradation of wood components*. Higuchi, T. Ed.; Publisher: Academy Press, New York, pp. 209-288.
- Hoong, Y.B., Paridah, M.T., Loh, Y.F., Jalaluddin, H., Chuah, L.A. (2011). A new source of natural adhesive: Acacia mangium bark extracts co-polymerized with phenol-formaldehyde (PF) for bonding Mempisang (*Annonaceae* spp.) veneers. *Int. J. Adhes. Adhes.* 31: 164-167.
- Howard, E.T. (1971). Bark structure of the southern pines. *Wood Sci.* 3: 134-148.
- Hümmer, W., Schreier, P. (2008). Analysis of proanthocyanidins. *Mol. Nutr. Food Res.* 52: 1381-1398.
- Inoue, S., Asaga, M., Ogi, T., Yazaki, Y. (1998). Extraction of polyflavonoids from radiata pine bark using hot compressed water at temperatures higher than 100°C. *Holzforschung* 52: 139-145.
- Ishimaru, K., Nonaka, G.-I., Nishioka, I. (1987). Flavan-3-ol and procyanidin glycosides from quercus miyagii. *Phytochemistry* 26: 1167-1170.
- Jerez, M., Selga, A., Sineiro, J., Torres, J.L., Núñez, M.J. (2007a). A comparison between bark extracts from *Pinus pinaster* and *Pinus radiata*: antioxidant activity and procyanidin composition. *Food Chem.* 100: 439-444.
- Jerez, M., Touriño, S., Sineiro, J., Torres, J.L., Núñez, M.J. (2007b). Procyanidins from pine bark: Relationships between structure, composition and antiradical activity. *Food Chem.* 104: 518-527.
- Jerez, M., Sineiro, J., Núñez, M.J. (2009a). Fractionation of pine bark extracts: selecting procyanidins. *Eur. Food Res. Technol.* 229: 651-659.
- Jerez, M., Sineiro, J., Guitián, E., Núñez, M.J. (2009b). Identification of polymeric procyanidins from pine bark by mass spectrometry. *Rapid Commun. Mass Spectrom.* 23: 4013-4018.
- Jorge, F.C., Brito, P., Pepino, L., Portugal, A., Gil, M.H., Irle, M.A., da Costa, R.P. (1999). Improved extraction of pine bark for wood adhesives. *Proceedings of the 3rd European Panel Product Symposium, Llandudno, Wales*; pp. 301-307.
- Karonen, M., Loponen, J., Ossipov, V., Pihlaja, K. (2004). Analysis of procyanidins in pine bark with reversed-phase and normal-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. *Anal. Chim. Acta.* 522: 105-112.
- Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.-P., Pihlaja, K., Kujala, T.S., Heinonen, M. (2011). Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* 47: 3954-3962.
- Kain, G., Güttler, V., Barbu, M.-C., Petutschnigg, A., Richter, K., Tondi, G. (2014). Density related properties of bark insulation boards bonded with tannin hexamine resin. *Eur. J. Wood Prod.* 72: 417-424.

- Kemppainen, K., Siika-aho, M., Pattathil, S., Giovando, S., Kruus, K. (2014). Spruce bark as an industrial source of condensed tannins and non-cellulosic sugars. *Ind. Crop. Prod.* 52: 158-168.
- Kim, S., Kim, H.J. (2004). Evaluation of formaldehyde emission of pine and wattle tannin-based adhesives by gas chromatography. *Holz Roh. Werkst.* 62: 101-106.
- Koch, G., Kleist, G. (2001). Application of Scanning UV Microspectrometry to Localise Lignin and Phenolic Extractives in Plant Cell Walls. *Holzforschung* 55: 563-567.
- König, B., Roffael, E. (2002). Zur Reaktivität von Tanninen in Abhängigkeit vom Kambialalter. *Holz Roh. Werkst.* 60: 258.
- König, B., Roffael, E. (2003). Zur Extraktion von Fichtenrinde mit Harnstoff und Harnstoff-Formaldehyde-Polymeren. *Holz Roh. Werkst.* 61: 206-212.
- Kraus, T.E.C., Zu, Z., Preston, C.M., Dahlgren, R.A., Zasoski, R.J. (2003). Linking chemical reactivity and protein precipitation to structural characteristics of foliar tannins. *J. Chem. Ecol.* 29: 703-730
- Krekling, T., Franceschi, V.R., Berryman, A.A., Christiansen, E. (2000). The structure and development of polyphenolic parenchyma cells in Norway spruce (*Picea abies*) bark. *Flora* 195: 354-369.
- Krizaj, B., Stupar, J. (1996). Potassium content in living bark, cambium and wood in relation to electrical resistance and tree condition in the Silver fir (*Abies alba* Mill.). *Phyton: Int. J. Exp. Bot.* 36: 39-42.
- Krogell, J., Holmbom, B., Pranovich, A., Hemming, J., Wilfor, S. (2012). Extraction and chemical characterization of Norway spruce inner and outer bark. *Nord. Pulp Pap. Res. J.* 27: 6-17.
- Labosky, P. (1979). Chemical-constituents of 4 Southern pine barks. *Wood Science* 12: 80-85.
- Lacoste, C., Čop, M., Kemppainen, K., Giovando, S., Pizzi, A., Laboire, M.P., Sernek, M., Celzard, A. (2015). Biobased foams from condensed tannin extracts from Norway spruce (*Picea abies*) bark. *Ind. Crop. Prod.* 73: 144-153.
- Laks, P.E. (1991). Chemistry of bark. In: Hon, D.N.-S. and Shirashi, N., Eds. *Wood and cellulosic chemistry* (1st Ed.); Marcel Dekker, New York; p. 257-330.
- Latva-Mäenpää, H., Laakso, T., Sarjala, T., Wähälä, K., Saranpää, P. (2013). Variation of stilbenes glucosides in bark extracts obtained from roots and stumps of Norway spruce (*Picea abies* [L.] Karst.). *Trees* 27: 131-139.
- Le Normand, M., Edlund, U., Holmbom, B., Ek, M. (2012). Hot-water extraction and characterization of spruce bark non-cellulosic polysaccharides. *Nord. Pulp Paper Res.* 27: 18-23.
- Le Normand, M., Mélida, H., Holmbom, B., Michaelsen, T.E., Inngjerdigen, M., Bulone, V., Paulsen, B.S., Ek, M. (2014). Hot-water extracts from the inner bark of Norway spruce with immunomodulating activities. *Carbohydr. Polym.* 101: 699-704.
- Li, J., Maplesden, F. (1998). Commercial production of tannins from radiata pine bark for wood adhesives. *IPENZ transaction* 25: 46-52.
- Li, S.-H., Niu, X.-M., Zahn, S., Gershenzon, J., Weston, J., Schneider, B. (2008). Diastomeric stilbene glucoside dimers from the bark of Norway spruce (*Picea abies*). *Phytochemistry* 69: 772-782.
- Liang, S., Guo, X.Y., Lautner, S., Saake, B. (2014). Removal of hexavalent chromium by different modified spruce bark adsorbents. *J. Wood Chem. Technol.* 34: 273-290.
- Liiri, O., Sairanen, H., Kilpeläinen, H., Kivistö, A. (1982). Bark extractives from Spruce as constituents of Plywood Bonding Agents. *Holz Roh. Werkst.* 40: 51-60.
- Maie, N., Behrens, A., Knicker, H., Knaber-Kögel, I. (2003). Changes in the structure and protein binding ability of condensed tannins during decomposition of fresh needles and leaves. *Soil Biol. Biochem.* 35: 577-589.
- Mannila, E., Talvitie, A. (1992). Stilbenes from *Picea abies* bark. *Phytochemistry* 31: 3288-3289.

- Manns, D., Deuschle, A.L., Saake, B., Meyer, A.S. (2014). Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae). *RSC Advanced*, 4: 25736-25746.
- Martin-Dupont, F., Gloaguen, V., Granet, R., Guilloton, M., Morvan, H., Krausz, P. (2011). Heavy metal adsorption by crude coniferous barks: a modelling study. *J. Environ. Sci. Heal. A* 37: 1063-1073.
- Matamala, G., Smeltzer, W., Droguett, G. (2000). Comparison of steel anticorrosive protection formulated with natural tannins extracted from acacia and from pine bark. *Corrosion Sci.* 42: 1351-1362.
- Matthews, S., Mila, I., Scalbert, A., Donnelly, D.M.X. (1997a). Extractable and non-extractable proanthocyanidins in barks. *Phytochemistry* 45: 405-410.
- Matthews, S., Mila, I., Scalbert, A., Pollet, B., Lapiere, C., Hervé du Penthoat, C.L.M., Rolando, C., Donnelly, D.M.X. (1997b). Method for estimation of proanthocyanidins based on their acid depolymerization in the presence of nucleophiles. *J. Agric. Food Chem.* 45: 1195-1201.
- Meiklahem, N., Pizzi, A., Stephanou, A. (1994). Induced accelerated autocondensation of polyflavonoid tannins for phenolic polycondensates. I. ¹³C-NMR, ²⁹Si-NMR, X-Ray, and polarimetry studies and mechanism. *J. Appl. Pol. Sci.* 54: 1827-1845.
- Miranda, I., Gominho, J., Mirra, I., Pereira, H. (2012). Chemical characterization of barks from *Picea abies* and *Pinus sylvestris* after fractioning into different sizes. *Ind. Crop. Prod.* 36: 395-400.
- Mun, S.P., Ku, C.S. (2006). Characterization of low molecular weight polyphenols from pine (*Pinus radiata*) bark. *Food Sci. Biotechnol.* 15: 424-430.
- Murray, N.J., Williamson, M.P., Lilley, T.H., Haslam, E. (1994). Study on the interaction between salivary prolin-rich proteins and a polyphenol by ¹H-NMR spectroscopy. *Eur. J. Biochem.* 219: 923-935.
- Musha, Y., Goring, D.A.I. (1975). Distribution of syringyl and guaiacyl moieties in hardwoods as indicated by ultraviolet microscopy. *Wood Sci. Technol.* 9:45-58.
- Myers, R.H., Montgomery, D.C., Anderson-Cook, C.M. (2009). *Response surface methodology: Process and Product Optimization Using Designed Experiments*. 3rd Edition. Publisher: John Wiley and Sons, Hoboken, New Jersey.
- Navarrete, P., Pizzi, A., Pasch, H., Rode, K., Delmotte, L. (2010). MALDI-TOF and ¹³C NMR characterization of maritime pine industrial tannin extract. *Ind. Crop. Prod.* 32: 105-110.
- Navarrete, P., Pizzi, A., Bertaud, E., Rigolet, S. (2011). Condensed tannin reactivity inhibition by internal rearrangements: detection by CP-MAS ¹³C NMR. *Maderas Cienc. Tecnol.* 13: 59-68.
- Norris C.E., Preston, C.M., Hogg, K.E., Titus, B.D. (2011). The influence of condensed tannin structure on rate of microbial mineralization and reactivity to chemical assay. *J. Chem. Ecol.* 37: 311-319.
- Nunes, E., Quilhó, T., Pereira, H. (1999). Anatomy and chemical composition of *Pinus pinea* L. bark. *Ann. For. Sci.* 56: 479-484.
- Packer, L., Rimbach, G., Virgili, F. (1999). Antioxidant activity and biological properties of a procyanidins-rich extracts form pine (*Pinus maritima*) bark, pycnogenol. *Free Radic. Biol. Med.* 27: 704-724.
- Pan, H. (1995). Studies on phenolic and terpenoid constituents extracted from bark of birch, spruce and pine in Sweden. PhD dissertation. Swedish University of Agricultural Science, Uppsala, Sweden.
- Parameswaran, N., Kruse, J., Liese, W. (1976). Aufbau und Feinstruktur von Periderm und Lenticellen der Fichtenrinde. *Z. Pflanzenphysiol.* 77: 212-221.
- Pasch, H., Pizzi, A., Rode, K. (2001). MALDI-TOF mass spectrometry of polyflavonoid tannins. *Polymer* 42: 7531-7539.

- Pasch, H., Pizzi, A. (2002). Considerations on the macromolecular structure of chestnut ellagitannins by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. *J. Appl. Polym. Sci.* 85: 429-437.
- Pichelin, F., Kamoun, C., Pizzi, A. (1999). Hexamine hardener behaviour: effects on wood glueing, tannin and other wood adhesives. *Holz Roh. Werkst.* 57: 305-317.
- Pichelin, F., Nakatani, M., Pizzi, A., Wieland, S., Despres, A., Rigolet, S. (2006). Structural beams from thick wood panels bonded industrially with formaldehyde-free tannin adhesives. *Forest Prod. J.* 56: 31-36.
- Pietarinen, S.P., Willför, S.M., Ahotupa, M.O., Hemming, J.E., Holmbom, B.R. (2006). Knotwood and bark extracts: strong antioxidants from waste materials. *J. Wood Sci.* 52: 436-444.
- Pizzi, A. (1982a). Condensed tannins for adhesives. *Ing. Eng. Chem. Prod. Res. Dev.* 21: 359-369
- Pizzi, A. (1982b). Pine tannin adhesives for particleboard. *Holz Roh. Werkst.* 40: 293-298.
- Pizzi, A., Tekely, P. (1995). Mechanism of polyphenolic tannin resin hardening by hexamethylenetetramine: CP-MAS ¹³C-NMR. *J. App. Pol. Sci.* 56: 1645-1650.
- Pizzi, A., Meikleham, N., Dombo, B., Roll, W. (1995). Autocondensation-based, zero-emission, tannin adhesives for particleboard. *Holz Roh. Werk.* 53: 201-204.
- Pizzi, A. (2008). Tannins: major sources, properties and applications, in: *Monomers, Polymers and Composites from Renewable Sources*; Belgacem, M.N., Gandini, A., Eds.; Elsevier, Oxford, UK; pp. 179-199.
- Pizzi, A., Stephanou, A. (1994a). A ¹³C NMR Study of Polyflavonoid Tannin Adhesive Intermediates. I. Noncolloidal Performance Determining Rearrangements. *J. Appl. Polym. Sci.* 51: 2109-2124.
- Pizzi, A., Stephanou, A. (1994b). Fast vs. slow-reacting non-modified tannin extracts for exterior particleboard adhesives. *Holz Roh. Werkst.* 52: 218-222.
- Porter, L.J. (1989). Condensed Tannins. in: *Natural Products of Woody Plants*; Rowe, J.W. Ed., Springer-Verlag, Berlin, Germany; vol. 1, pp. 651-690.
- Porter, L.J. (1992). Structure and chemical properties of the condensed tannins. In: Hemingway, R.W., Laks, P.E. (Eds.), *Plant Polyphenols, Basic Life Sciences*, vol. 59. Springer Science + Business Media, New York, pp. 245-258.
- Prislan, P., Koch, G., Schmitt, U., Gričar, J., Čufar, K. (2012). Cellular and topochemical characteristics of secondary changes in bark tissues of beech (*Fagus sylvatica*). *Holzforschung* 66: 131-138.
- Putoczki, T.L., Pettolino, F., Griffin, M.D.W., Möller, R., Gerrard, J.A., Bacic, A., Jackson, S.L. (2007). Characterization of the structure, expression and function of *Pinus radiata* D.Don arabinogalactan-proteins. *Planta* 226: 1131-1142.
- Roffael, E., Dix, B., Okum, J. (2000). Use of spruce tannin as binder in particleboards and medium density fiberboards (MDF). *Holz Roh. Werkst.* 58: 301-305.
- Ross, T.K., Francis, R.A. (1978). Treatment of rusted steel with mimosa tannin. *Corrosion Sci.* 18: 351-361.
- Rothpfeffer, C., Karlton, E. (2007). Inorganic elements in tree compartments of *Picea abies* - concentrations versus stem diameter in wood and bark and concentration in needles and branches. *Biomass Bioener.* 31: 717-725.
- Saarela, K.E.; Harju, L.; Lill, J.O.; Heselius, S.J.; Rajander, J.; Lindroos, A. (2005). Quantitative elemental analysis of dry-ashed bark and wood samples of birch, spruce and pine from South-Western Finland using PIXE. *Acta Academie Aboensis, Ser.B Vol.* 64 nr 1.
- Sakai, K. (2001). Chemistry of bark. In: *Wood and cellulosic chemistry*. Hon, D.N.-S. and Shirashi, N., Eds.; Marcel Dekker, New York; p. 243-273.

- Sánchez-Martín, J., Beltrán-Heredia, J., Gibello-Pérez, P. (2011). Adsorbent biopolymers from tannin extracts for water treatment. *Chem. Eng. J.* 168: 1241-1247.
- Sarneckis, C.J., Dambergs, R.G., Jones, P., Mercurio, M., Herderich, M.J., Smith, P.A. (2006). Quantification of condensed tannins by precipitation with methylcellulose: development and validation of an optimised tool for grape and wine analysis. *Aust. J. Grape Wine R.* 12: 39-49.
- Sauget, A., Nicollin, A., Pizzi, A. (2013). Fabrication and mechanical analysis of mimosa tannin and commercial flax fibers biocomposites. *J. Adhes. Sci. Technol.* 27: 2204-2218.
- Scalbert, A., Mila, I., Expert, D., Marmolle, F., Albrecht, A.-M., Hurrell, R., Huneau, J.-F., Tomé, D. (1999). Polyphenol, metal ion complexation and biological consequences. In: *Plant polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*. Eds: Gross et al. Kluwer Academic / Plenum Publisher, New York.
- Schofield, J.A., Hagerman, A.E., Harold, A. (1988). Loss of tannins and other phenolics from willow leaf litter. *J. Chem. Ecol.* 24: 1409-1421.
- Sealy-Fisher, V.J., Pizzi, A. (1992). Increased pine tannins extraction and wood adhesives development by phlobaphenes minimization. *Holz Roh. Werkst.* 50: 212-220.
- Seki, K., Saito, N., Aoyama, M. (1997). Removal of heavy metal ions from solutions by coniferous barks. *Wood Sci. Technol.* 31: 441-447.
- Shen, Z., Haslam, E., Falshaw, C.P., Begley, M.J. (1986). Procyanidins and polyphenols of *Larix gmelinii* bark. *Phytochemistry* 25: 2696-2635.
- Singelton, V.L., Orthofer, R., Lamuela-Raventos, R.M. (1999). Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. *Method Enzymol.* 299: 152-178.
- Sinkkonen, J., Karonen, M., Liimatainen, J., Pihlaja, K. (2006). Lignans from the bark extract of *Pinus sylvestris* L. *Magn. Reson. Chem.* 44: 633-636.
- Solhaug, K.A. (1990). Stilbene glucosides in bark and needles from *Picea* species. *Scand. J. For. Res.* 5: 59-67.
- Stafford, H.A., Cheng, T.Y. (1980). Procyanidins of Douglas-fir seedlings, callus and cell-suspension cultures derived from cotyledons. *Phytochemistry* 19: 131-135.
- Stafford, H.A. (1988). Proanthocyanidins and the lignin connection. *Phytochemistry* 27: 1-6
- Steenkamp, J.A., Steynberg, J.P., Brandt, E.V., Ferreira, D. Roux, D.G. (1985). Phlobatannins, a novel class of ring-isomerized condensed tannins. *J. Chem. Soc., Chem. Comm.* 1678.
- Steynberg, P.J., Steynberg, J.P., Bezuidenhout B.C.B., Ferreira, D. (1994). Cleavage of the interflavanil bond in 5-deoxy (A-ring) proanthocyanidins. *J. Chem. Soc., Chem. Comm.*, 1: 31-32.
- Sun, B., Leandro, C., da Silva, J.M.R., Spranger, I. (1998). Separation of grape and wine proanthocyanidins according to their degree of polymerization. *J. Agr. Food Chem.* 46: 1390-1396.
- Takagaki, A., Fukai, K., Nanjo, F., Hara, Y. (2000). Reactivity of green tea catechin with formaldehyde. *J. Wood Sci.* 46: 334-338.
- Tiarks, A.E., Bridges, J.R., Hemingway, R.W., Shoulders, E. (1989). Condensed tannins in southern pines and their interaction with the ecosystem. In: Hemingway, R.W., Karchesy, J.J. (Eds.), *Chemistry and Significance of Condensed Tannins*. Plenum Press, New York, pp. 369-390.
- Timell, T.E. (1961). Isolation of polysaccharides from the bark of gymnosperms. *Sven. Papperstidn.* 64: 651-661
- Tisler, V., Galla, E., Pulkkinen, E. (1986). Fractionation of hot water extracts from *Picea abies* Karst. bark. *Holz Roh. Werk.* 44: 427-431.

- Tondi, G., Pizzi, A. (2009). Tannin-based rigid foams: a survey of chemical and physical properties. *Bioresource Technol.* 100: 5162-5169.
- Toscano Underwood, C.D., Pearce, R.B. (1991). Astringin and isorhapontin distribution in Sitka spruce trees. *Phytochemistry* 30: 2183-2189.
- Trosa, A., Pizzi, A. (2001). A no-aldehyde emission hardener for tannin-based wood adhesives for exterior panels. *Holz Roh. Werk.* 59: 266-271.
- Ucar, M.B., Ucar, G., Pizzi, A., Gonultas, O. (2013). Characterization of *Pinus brutia* bark tannin by MALDI-TOF MS and ¹³C NMR. *Ind. Crop. Prod.* 49: 697-704.
- Vane, C.H., Drage, T.C., Snape, C.E. (2006). Bark decay by white-rot fungus *Lentinula edodes*: polysaccharide loss, lignin resistance and the unmasking of suberin. *Int. Biodeter. Biodeg.* 57: 14-23.
- Valentin, L., Kluczek-Turpeinen, B., Willför, S., Hemming, J., Hatakka, A., Steffen, K., Tuomela, M. (2010). Scots pine (*Pinus sylvestris*) bark composition and degradation by fungi: Potential substrate for bioremediation. *Biores. Technol.* 101: 2203-2209.
- Valenzuela, J., Von Leyser, E.P., Pizzi, A., Westermeyer, C., Gorrini, B. (2012). Industrial production of pine tannin-bonded particleboard and MDF. *Eur. J. Wood Wood Prod.* 70: 735-740.
- Vázquez, G., González-Alvarez, J., Freire, S., López-Suevos, F., Antorrena, G. (2001). Characteristics of *Pinus pinaster* bark extracts obtained under various extraction conditions. *Holz Roh. Werkst.* 59: 451-456.
- Vázquez Penas, D., Lage Yusty, M.A., Parajó Liñares, J.C. (1992). Chemical-biotechnological processing of pine bark. *J. Chem. Tech. Biotechnol.* 54: 63-74.
- Venter, P.B., Sisa, M., van der Merwe, M.J., Bonnet, S.L., van der Westhuizen, J.H. (2012a). Analysis of commercial proanthocyanidins. Part 1: The chemical composition of quebracho (*Schinopsis lorenzii* and *Schinopsis balansae*) heartwood extract. *Phytochemistry* 73: 95-105
- Venter, P.B., Senekal, N.D., Kemp, G., Amra-Jordaan, M., Khan, P., Bonnet, S.L., van der Westhuizen, J.H. (2012b). Analysis of commercial proanthocyanidins. Part 3: The chemical composition of wattle (*Acacia mearnsii*) bark extract. *Phytochemistry* 83: 153-167.
- Weissman, G. (1981). Untersuchung der Rinderextrakte von *Picea abies* Karst. *Holz Roh. Werkst.* 39: 457-461.
- Weissman, G. (1983). Studies on pine bark extracts. *Int. J. Adhes. Adhes.* 3: 31-35.
- Weissman, G. (1984). Zusammensetzung der wässrigen Rindenextrakte von *Picea abies* [Karst.] im Verlauf einer Vegetationsperiode. *Holzforschung* 38: 7-10.
- Weissman, G. (1985a). Untersuchung der Rinderextrakte von Lärchen. *Holzforsch. Holzwert.* 37: 67-71.
- Weissman, G. (1985b). Analyse von sauren und neutralen Zuckern in Rindenextrakten von *Pinus radiata* und *Larix decidua* durch Kapillar-Gaschromatographie. *Holzforschung* 39: 245-247.
- Wetzel, S., Greenwood, J.S. (1989). Proteins as a potential nitrogen storage compound in bark and leaves of several softwoods. *Trees* 3; 149-153.
- Willför, S., Pranovich, A., Tamminen, T., Puls, J., Laine, C., Suurnäkki, A., Saake, B., Uotila, K., Simolin, H., Hemming, J., Holmbom, B. (2009). Carbohydrate analysis of plant materials with uronic acid-containing polysaccharides – A comparison between different hydrolysis and subsequent chromatographic analytical techniques. *Ind. Crop. Prod.* 29: 571-581.
- Xing, C., Deng, J., Zheng, S.Y., Riedl, B., Cloutier, A. (2006). Impact of bark content on the properties of medium density fibreboard (MDF) in four species grown in eastern Canada. *Forest Prod. J.* 56: 64-69
- Ya, C., Gaffney, S.H., Lilley, T.H., Haslam, E. (1989). Carbohydrate-polyphenol complexation. In: Hemingway, R.W., Karchesy, J.J. (Eds.), *Chemistry and Significance of Condensed Tannins*. Plenum Press, New York, pp. 307-322.

- Yamaguchi, H., Higashida, R., Higuchi, M., Sakata, I. (1992). Adsorption mechanism of heavy-metal ion by microspherical tannin resin. *J. Appl. Polym. Sci.* 45: 1463-1473
- Yazaki, Y. (1980). Improved ultrafiltration of extracts from *Pinus radiata* bark. *Holzforschung* 39: 79-83.
- Yazaki, Y. (1985). Extraction of polyphenols from *Pinus radiata* bark. *Holzforschung* 39: 267-271.
- Yazaki, Y. (1987). Solubility of extracts from *Pinus radiata* bark. *Holzforschung* 41: 23-26.
- Yazaki, Y., Aung, T. (1988). Alkaline extraction of *Pinus radiata* bark and isolation of aliphatic dicarboxylic acids. *Holzforschung* 42: 357-360.
- Yazaki, Y., Hillis, W.E. (1977). Polyphenolic extractives of *Pinus radiata* bark. *Holzforschung* 31: 20-25.
- Yazaki, Y., Hillis, W.E. (1980). Molecular size distribution of *Radiata* pine bark extracts and its effect on properties. *Holzforschung* 34: 125-130.
- Yazaki, Y., Collins, P.J. (1994). Wood adhesives based on tannin extracts from barks of some pine and spruce species. *Holz Roh. Werkst.*, 52: 307-310.
- Yesil-Celiktas, O., Otto, F., Parlar, H. (2009a). A comparative study of flavonoid contents and antioxidant activities of supercritical CO₂ extracted pine barks grown in different regions of Turkey and Germany. *Eur. Food Res. Technol.* 229: 671-677.
- Yesil-Celiktas, O., Ganzera, M., Akgun, I., Sevimli, C., Korkmaz, K.S., Bedir, E. (2009b). Determination of polyphenolic constituents and biological activities of bark extracts from different *Pinus* species. *J. Sci. Food Agric.* 89: 1339-1345.
- Yoneda, S., Nakatsubo, F. (1998). Effects of the hydroxylation patterns and degrees of polymerization of condensed tannins on their metal-chelating capacity. *J. Wood Chem. Technol.* 18: 193-205.
- Zhang, L., Gellerstedt, G. (2008). 2D heteronuclear (1H-13C) Single Quantum Correlation (HSQC) NMR Analysis of Norway Spruce Bark Components, in: Hu, T.Q. (Ed.). *Characterization of Lignocellulosic Materials*; Publisher: Blackwell Publishing Ltd. Oxford, UK; pp. 3-12.
- Zhiqun, L., Xiaoping, L., Renshu, L., Xiangly, W. (1994). Larch bark tannin adhesive for exterior particleboard. *J. Northeast For. Univ.* 5: 79-82.
- Zhou, H.-C., Tam, N.F.-Y., Lin, Y.-M., Wei, S.-D., Li, Y.-Y. (2012). Changes of condensed tannins during decomposition of leaves of *Kandelia obovata* in subtropical mangrove swamp in China. *Soil Biol. Biochem.* 44: 113-121.
- Zucker, W.V. (1983). Tannins: does structure determine function? An ecological perspective. *Am. Nat.* 121: 335-365.

7. Index of figures

Figure 1:	Schematic molecular structure of some hydrolysable tannins	6
Figure 2:	Schematic molecular structure of condensed tannins	7
Figure 3:	Parenchyma cells in the secondary phloem of Norway spruce	9
Figure 4:	Most typical pyran ring rearrangements of a procyanidin dimer	13
Figure 5:	Schematic structure of softwood outer bark	16
Figure 6:	Molecular structure of some phenolic monomers detected in softwood barks	20
Figure 7:	Schematic structure of a softwood bark arabinan	24
Figure 8:	Viscosity of aqueous solutions of crude and purified bark extract from <i>Radiata</i> pine and European larch	29
Figure 9:	Flowchart of a tannin extraction plant with 4 successive extraction stages	31
Figure 10:	Sampling of Norway spruce bark flakes using a debarking iron	33
Figure 11:	Freeze dried hot water extracts from the bark of a) Silver fir, b) European larch, c) Norway spruce, d) Douglas fir, e) Scots pine.	35
Figure 12:	Scheme of the analytical procedure applied for the analysis of the bark and bark extracts	36
Figure 13:	SEM pictures of the bark of different softwood species	40
Figure 14:	UV-absorbance maps (280 nm) of Norway spruce bark transverse sections	44
Figure 15:	Point UV-absorbance spectra of different anatomical spots in Norway spruce bark	44
Figure 16:	HPLC-UV chromatographs before and after thiolysis of Norway spruce crude extract and its fractions F0, F1 and F2	47
Figure 17:	HPLC-UV chromatogram (25-50 min, left) and MALDI-TOF mass spectrum (300-900 m/z, right) of Silver fir bark crude extracts	52
Figure 18:	HPLC-UV chromatogram (25-50 min - left) and MALDI-TOF mass spectrum (300-900 m/z - right) of European larch bark crude extracts	52
Figure 19:	HPLC-UV chromatogram (25-50 min - left) and MALDI-TOF mass spectrum (300-900 m/z - right) of Norway spruce bark crude extracts	53
Figure 20:	HPLC-UV chromatogram (25-50 min - left) and MALDI-TOF mass spectrum (300-900 m/z - right) of Douglas fir crude bark extracts	53

Figure 21:	HPLC-UV chromatogram (25-50 min - left) and MALDI-TOF mass spectrum (300-900 m/z - right) of Scots pine crude bark extracts	54
Figure 22:	MALDI-TOF mass spectra (900-4000 m/z) of Silver fir bark extracts	56
Figure 23:	MALDI-TOF mass spectra (900-4000 m/z) of European larch bark extracts	56
Figure 24:	MALDI-TOF mass spectra (900-4000 m/z) of Norway spruce bark extracts	57
Figure 25:	MALDI-TOF mass spectra (900-4000 m/z) of Douglas fir bark extracts	57
Figure 26:	MALDI-TOF mass spectra (900-4000 m/z) of Scots pine bark extracts	58
Figure 27:	Likely structure of a Norway spruce bark tannin containing an astringin dimer as a terminal unit.	59
Figure 28:	Total extraction yield of Norway spruce bark ground to different sizes	63
Figure 29:	SEM pictures of Norway spruce bark ground to different size	63
Figure 30:	UV-absorbance spectra of phenolic deposits of Norway spruce bark at different depths from the surface	64
Figure 31:	Response surface plots for total, phenolic, tannin and carbohydrate yields as a function of extraction temperature and time	67
Figure 32:	Response surface plots for total, phenolic, tannin and carbohydrate yields as a function of extraction temperature and concentration of sulfites in the extraction water	68
Figure 33:	Total and phenolic extraction yield of Norway spruce and Scots pine bark in sequential extractions at increasing temperature	69
Figure 34:	Phenolic composition of Norway spruce bark extracts from successive extractions at increasing temperature	70
Figure 35:	MALDI-TOF mass spectra of Norway spruce bark extracts obtained from successive extractions at increasing temperature	72
Figure 36:	Total extraction yield and concentration of phenolic compounds in the extracts during outdoor storage of Norway spruce bark	74
Figure 37:	HPLC-UV chromatograms of Norway spruce extracts from samples collected at different bark weathering time	75
Figure 38:	MALDI-TOF mass spectra of Norway spruce bark extracts from samples collected at 1 and 210 days of outdoor storage	76

8. Index of tables

Table 1:	Extraction yields of successive extractions of softwood barks with solvents at increasing polarity	19
Table 2:	Scheme of the process used for the characterization of the total extractives in softwood barks	34
Table 3:	Total extractives and total condensed tannins in softwood bark	41
Table 4:	Carbohydrate monomeric composition in softwood bark	43
Table 5:	Total yield of softwood bark extracts and their phenolic, carbohydrate and inorganic (ashes) compositions	45
Table 6:	Phenolics' and tannins' yields from hot water extractions of softwood barks	48
Table 7:	Total, free and bound carbohydrates in softwood bark extracts	49
Table 8:	Structural characteristics determined by thiolysis and HPLC-UV of softwood bark condensed tannins in crude hot water extracts and extract fractions F1 and F2	55
Table 9:	Monomeric composition of carbohydrates in softwood bark crude extracts and extract fractions F1 and F2	61
Table 10:	Experimental design performed on the hot water extraction of Norway spruce bark	65
Table 11:	Significances (p-value) and regression coefficients (β) of the experimental design performed on hot water extraction of Norway spruce bark	66
Table 12:	Composition of total, free and bound carbohydrates in Norway spruce and Scots pine bark extracts by successive extractions at increasing temperatures	71
Table 13:	Extracted phenolics and total, extracted and non-extractable procyanidins of Norway spruce bark samples after different durations of storage	73
Table 14:	Composition of total, free and bound carbohydrates in Norway spruce extracts from samples collected at different bark weathering time.	77