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Report on the Extractives Composition of Each Feedstock

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Report on the Extractives Composition of Each Feedstock

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Executive Summary

This deliverable presents data obtained for the extractives contents of a range of samples considered to be of relevance to the UNRAVEL project, its FABIOLA pre-treatment process, and the interests of the partners of the consortium. There is a particular focus on forestry hardwoods, with three species (birch, beech, and poplar) investigated. For each of those species separate anatomical fractions of the plant are analysed (bark, foliage, branches, stem wood, and foliage). Additionally, a number of straw samples are analysed as well as two grasses (roadside grass and switchgrass) and two samples of olive-tree prunings.

In the context of the UNRAVEL project extractives contents are considered to be important for two main reasons. Firstly, it has previously been shown through exploratory work at ECN that these extractives can interfere with the mechanisms of the FABIOLA fractionation (pre-treatment) process and can lead to lower yields and lower purities of the targeted biomass fractions (cellulose, lignin, and monomeric hemicellulose sugars). Secondly, it has been hypothesised that some specific chemical constituents within the extracts may be of sufficiently high value, and be present in sufficiently-high concentrations, that their recovery may be warranted.

A literature review was undertaken to determine which specific compounds may be expected in the organic extracts of the UNRAVEL samples and, subsequently, a number of different extractions (using water, 95% ethanol, and acetone as solvents) were undertaken. The extractives contents associated with each extraction were calculated based on the solid mass loss, corrected for moisture content, that took place. The liquid extracts were also collected and stored with the water extracts analysed for carbohydrates (using ion chromatography) and the ethanol extracts analysed for a range of constituents (identified in the literature review) using HPLC coupled with MS and UV detection.

For all samples the full-extractives content (water followed by 95% ethanol extraction) was greatest, followed by the water extractives content, then the ethanol extractives content and, finally, the acetone extractives content. The acetone extractives contents are of particular relevance to FABIOLA process since it uses acetone for fractionation/pre-treatment. It was found that the difference (shortfall) of the acetone extractives contents, when compared with the ethanol extractives contents, increased with greater ethanol extractives contents.

With regards to the hardwood samples, it was found that the extractives contents were greatest for the bark and foliage samples whilst they were lowest in the debarked stem wood samples. The branch and non-debarked wood samples had intermediate extractive contents. It was concluded that, for these samples, pre-extraction (in order to improve the efficacy of subsequent FABIOLA fractionation) would only be warranted for bark and foliage samples meaning that direct pre-treatment of stem wood would be acceptable. In these samples there were appreciable levels of sugars in the water extract and, in the case of birch bark, significant amounts of betulin (8.8%) and betulinic acid (0.6%) in the ethanol extract. It was concluded that, for these samples, the subsequent work on the simplification of pre-extraction (Task 2.2.1) would focus on the effect of this on the sugars contents in the extract and on the betulin/betulinic acid content in the extract, as well as on the effects on amounts of total extractives removed. It was also concluded that UNRAVEL Task 2.2.2 (“Increased Purity of

Target Extractive Compounds”) would focus on increasing the purity of betulin and betulinic acid in the extract from birch bark.

With regards to the other samples that were analysed, significant quantities of full-extractives were found for the olive-pruning, straw, cocoa husks, and cow manure fibre samples. For the straw samples it was noted that the difference between the water extractives and ethanol-extractives contents was greatest. In some cases (e.g. for the corn stover residues) these water extracts contained significant amounts of sugars but for other straw (and grass) samples the detected amounts of water-soluble carbohydrates were low. It was noted, based on a literature review that was undertaken, that water-soluble carbohydrate contents are highly variable according to a plethora of factors (e.g. season, storage method, yield etc.) so no definitive conclusions regarding the levels that may be expected for a particular feedstock can be made based on the analysis of one sample. However, the relatively high amounts of trehalose present in the olive tree prunings were of interest.

For all of the non-hardwood samples it appears that a pre-extraction at least using water would be warranted in order to obtain a cleaner sample for subsequent FABIOLA fractionation. This would also ensure that few water-soluble carbohydrates are present in the liquid fraction from pre-treatment, hopefully minimising the amounts of sugar degradation products (e.g. hydroxymethylfurfural) that could interfere with fermentation of the monomers liberated from hemicellulose. However, the concentrations of valuable chemicals in the ethanol extract for all of the non-birch-bark samples were very low and it was concluded that no constituents (other than betulin and betulinic acid) were present in sufficient quantities to warrant their inclusion in Task 2.2.2.

Table of Contents

Executive Summary 3
1 Background 8
2 Literature Review 10
2.1 Betulin and Betulinic Acid 10
2.2 Gallic Acid 12
2.3 Catechin 12
2.4 p-Coumaric Acid 13
2.5 Acacetin 13
2.6 Chlorogenic Acid 14
2.7 Salicilin 14
2.8 Salicortin 14
2.9 Quercetin and Isoquercetin 15
3 Analytical Techniques Undertaken 15
3.1 Sample Preparation and NIR Scanning 15
3.2 Ash Content 15
3.3 Solvent Extraction of Samples 16
3.4 Analysis of Water Extracts for Carbohydrates 16
3.5 Analysis of Ethanol Extract 17
3.5.1 Sample Preparation 17
3.5.2 Preparation of Standards 17
3.5.3 Analysis of Ethanol Extracts using LC-MS QTOF 17
3.5.4 Analysis of Ethanol Extracts using HPLC-DAD-FLD 18
4 Results 19
4.1 Extractives Contents 19
4.1.1 Birch 19
4.1.2 Beech 20
4.1.3 Poplar 20
4.1.4 Olive Trees 21
4.1.5 Grasses 21
4.1.6 Straws 21
4.1.7 Other Samples 21
4.2 Ash Contents after Full Extraction 23
4.3 Carbohydrates in Water Extractives 24
4.4 Data from the HPLC Analysis of the Ethanol Extract 28

5 Conclusion 35
6 Acknowledgement 36
7 References 36

List of Tables

Table 1: List of the UNRAVEL samples whose extractives compositions were determined. 10

Table 2: Extractives contents of the UNRAVEL feedstock samples. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. . . 22

Table 3: Ash contents and the amount of ash retained in the biomass after full extraction. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. 23

Table 4: Results for the total sugars, sucrose, glucose, fructose and trehlose contents of the feedstock samples. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample. 25

Table 5: Results for the mannose, galactose, rhamnosae, xylose, and arabinose contents of the feedstock samples. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample. 26

Table 6: Results for the xylitol, sorbitol, and mannitol contents of the feedstock samples. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample. 27

Table 7: Data for the concentrations of a range of constituents, expressed in their percent contribution to the total dry mass of the sample, found in the HPLC analysis of the ethanol extracts. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample. 31

Table 8: Data for the concentrations of theogallin, epicatechin gallate, acacetin, abrectorin, and methyl gallate, expressed in their percent contribution to the total dry mass of the sample, found in the HPLC analysis of the ethanol extracts. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample. 32

Table 9: Data for the concentrations of chlorogenic acid, oleuropein, methyl oleuropein, isosalicin, and salicortin, expressed in their percent contribution to the total dry mass of the sample, found in the HPLC analysis of the ethanol extracts. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample 33

Table 10: Data for the concentrations of quercetin, luteolin, isoquercetin, aesculetin, and aesculin, expressed in their percent contribution to the total dry mass of the sample, found in the HPLC analysis of the ethanol extracts. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample 34

1 Background

The UNRAVEL project aims to develop advanced pre-treatment, separation and conversion technologies for complex lignocellulosic biomass to produce usable lignin fragments, and monomeric sugars from the cellulose and hemicellulose fraction suitable for biochemical conversions. The conceptual process flow of UNRAVEL, Figure 1, incorporates several stages in the fractionation and conversion of biomass. It includes stage(s) for the removal of non-structural components (extractives) from the biomass prior to the main pre-treatment step. This step, or sequence of steps, is referred to as “pre-extraction”.

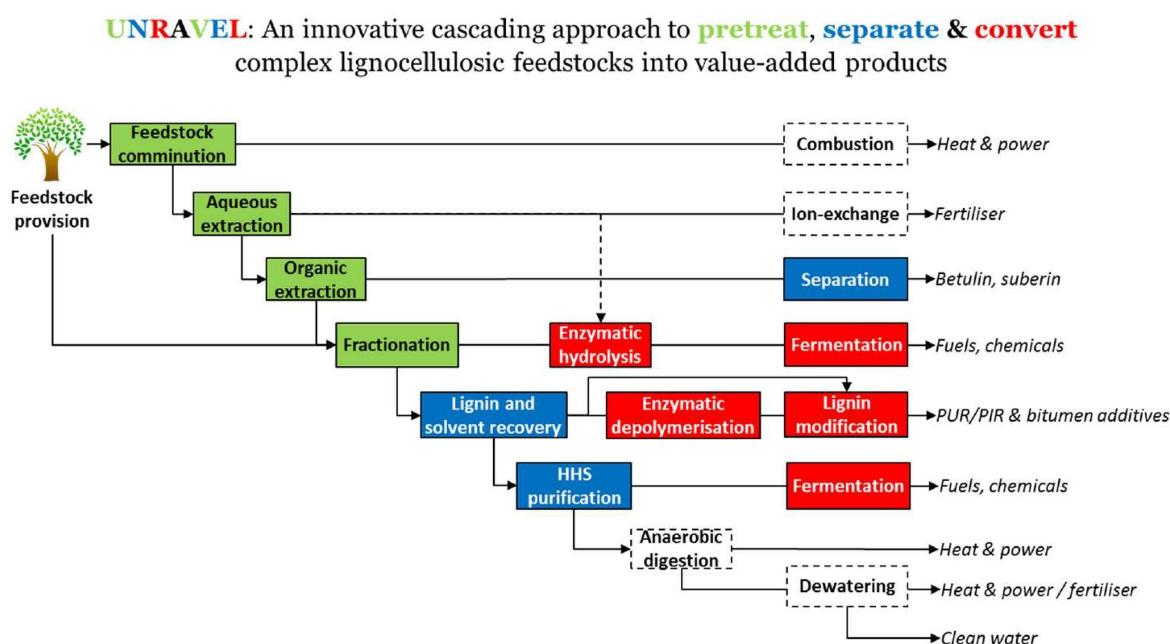

Figure 1: An overview of the UNRAVEL process.

Extractives are defined as extraneous components that may be separated from the insoluble cell wall material by their solubility in water or neutral organic solvents. There are a large number of different extractives, many of which are species-specific. These are often classified into categories according to the similarity of their chemical structures. Major categories include monosaccharides, polysaccharides, volatile oils, terpenes, fatty acids and their esters, waxes, polyhydric alcohols, alkaloids and aromatic compounds (Goldstein 1991). Many extractives have roles in the metabolic processes of a plant. The primary metabolites are inter-convertible biogenic intermediates and include monosaccharides, simple fats and various carboxylic acids. The more complex secondary metabolites tend to be irreversibly formed. These include sitosterol, simple terpenoids, chlorophyll, phenylpropanoids, the common flavenoids and simple tannins. Extractives play a wide array of functions in a plant, depending on their composition and location.

The first core objective of the UNRAVEL project concerns the removal of extractives, specifically to “recover valuable components from the feedstock and minimise the interference

of non-lignocellulosic constituents on the fractionation by developing a pre-extraction process optimised for mixed lignocellulosic biomass streams with complex composition".

Prior work at ECN on the FABIOLA pre-treatment found that pre-extraction prior to fractionation (pre-treatment) was essential for feedstocks with high extractives contents since these extractives, if not removed, lead to interferences in fractionation through undesired side-reactions between themselves and the structural lignocellulosic constituents of biomass (cellulose, hemicellulose, and lignin). Hence, fractionation without pre-extraction leads to lower yields and purity of the targeted lignocellulosic fractions. Conversely, when these components are fractionated from pre-extracted biomass not only were their yields and purities improved but there were also improvements in their properties (for example, better lignin properties and increased enzymatic cellulose digestibility).

Additionally, certain extractives may be of value for recovery, purification and sale. Task 2.2.2 of UNRAVEL concerns the "Increased Purity of Target Extractive Compounds". It targets, through lab-based experiments, a suitable strategy for recovering from the extracts of selected feedstocks, a selected subset of targeted high value extractive compounds with an appropriate purity and quality, while considering commercial viability and environmental aspects such as solvent and energy consumption.

In order to understand, for a specific feedstock, those extractives which may warrant recovery/purification, as well as those extractives that may cause issues in fractionation, detailed analysis of the extractives' composition is necessary. In Task 2, a total of 25 samples were analysed for their extractives content and lignocellulosic composition (data regarding the lignocellulosic composition were presented in Deliverable D.2.2). These samples covered a wide variety of different feedstock types and are listed in Table 1. An initial literature review was undertaken in order to understand the main extractives that would be expected in these samples, as well as their expected concentrations. These were used as guidelines for the purchase of relevant analytical standards and chromatography protocols for their identification and quantification in extracts. Subsequently, these samples were extracted using a number of different solvents (water, 95% ethanol, acetone) as well as with sequential extractions involving water followed by 95% ethanol. The water extracts were analysed for their carbohydrates, using ion chromatography equipment, and the ethanol extracts were analysed for a range of other constituents using HPLC equipment coupled with UV and/or MS detection.

Table 1: List of the UNRAVEL samples whose extractives compositions were determined.

Sample Type	Sample Code	Sample Name
Birch	BIC-1-SAP	SAPPI Birch Chips Batch 1
	BIC-1-TNO	Birch Stemwood Chips
	BIB-1-TNO	Birch Bark
	BIB-2-TNO	Birch Wood with Bark
	BIBR-1-TNO	Birch Branches
	BIF-1-TNO	Birch Foliage
Beech	BEC-1-TNO	Beech Stemwood Chips
	BEB-1-TNO	Beech Bark
	BEBR-1-TNO	Beech Branches
	BEF-1-TNO	Beech Foliage
Poplar	POC-1-TNO	Poplar Stemwood Chips
	POB-1-TNO	Poplar Bark
	POBR-1-TNO	Poplar Branches
	POF-1-TNO	Poplar Foliage
Olive Trees	OTR-1-TNO	Olive Tree Residues
	OTR-2-TNO	Intact Olive Branches
Grasses	RSG-1-TNO	Road Side Grass
	SWG-1-TNO	Switchgrass
Straws	WHS-1-TNO	Wheat Straw
	RIS-1-TNO	Rice Straw
	SFS-1-TNO	Sunflower Straw
	RSS-1-TNO	Rapeseed Straw
	COS-1-TNO	Corn Stover Residues
Other	CPH-1-TNO	Cocoa Pod Husk Batch 1
	CMF-1-TNO	Cow Manure Fibres

2 Literature Review

In UNRAVEL there is a particular focus on biomass obtained from hardwood trees as feedstock, due to the particular requirements of the core FABIOLA pre-treatment process and the interests of the consortium partners (e.g. SAPPI). There are also a few other key feedstocks of primary relevance to the project (e.g. wheat straw and municipal grass wastes). The literature review that was undertaken to investigate the expected extractives in the ethanolic extract focused on these key feedstocks. The identified chemical constituents are discussed below, along with observations on their relevant properties and on the potential applications of these extractive components.

2.1 Betulin and Betulinic Acid

Betulin is a naturally occurring terpene, more specifically it is a triterpene, containing three terpene units (or 6 isoprene units). Terpenes typically play a protectory role in plants. Betulin is isolated from the bark of birch trees, birch sap, *Inonotus obliquus* and red alder. In birch

betulin gives the outer part of the trunk its white colour which protects the tree from mid-winter overheating by the sun. Betulin can be converted to betulinic acid which is biologically more active than betulin. Betulinic acid differs from betulin in that it contains an extra oxygen atom, forming a carboxylic acid group.

In vitro studies have shown that betulin was effective against a variety of tumors by starting a process of malignant cells self-destruction called apoptosis (Zhao, Li et al. 2018). In vivo, betulin ameliorated diet-induced obesity, decreased the lipid contents in serum and tissues, and increased insulin sensitivity (Thounaojam, Nammi et al. 2016). Furthermore, betulin reduced the size and improved the stability of atherosclerotic plaques.

Betulinic acid has been found to have antiretroviral, antimalarial, and anti-inflammatory properties, as well as potential as an anticancer agent (Fulda 2008).

These high-value application of both betulin and betulinic acid suggest that these compounds could be worthy of purification from extracts, providing they are present in the biomass at sufficient concentrations. The price for betulin price can range from 300 USD/kg to 57,000 USD/kg based on purity (https://www.pharmacompass.com/price/betulin).

Birch Bark

The concentrations of betulin and betulinic acid in birch bark differ greatly according to the plant variety. For example, they are typically greatest in white birch (*Betula Platyphylla* Suk.). The concentration also varies according to the region, plant part, and plant age.

The extractives from Finnish birch were characterised by the Natural Resources Institute Finland and University of Eastern Finland under the project NORPYRO. According to the conference paper published by Roitto et al, (2015), the total mass percentage of extractives is double in branches and 5-10 times higher in bark compared to the stem biomass. In stem wood, only small amounts of betulinol and sterols were detected. Betulin concentration in bark was found to be 40-50 mg/g, in branches 4-5 mg/g and in stem it was only present in negligible concentrations. Betulin concentrations are also significantly different between plant species, for example, in *Birch pubescens*, average betulin concentrations in bark are two-fold higher (at approx 80 mg/g) than the betulin present in the bark of *Birch Pendula* (40 mg/g).

The triterpenoid composition in the outer bark of the dark-barked birch species *B.lenta* is significantly different from the white-barked species. Betulin is the predominant triterpene in white-bark species, whereas in black birch, lupeol is the major triterpene (Cole, Bentley et al. 1991).

Guo *et al*. (2017) analysed birch bark collected from 48 sites in Northeast China and found that the betulin concentration ranged from 16.97% to 34.3% (with an average of 23.91%) and that the betulinic acid concentration ranged from 1.31% to 4.54% (with an average of 2.89%). Zhao *et al*. (2007) examined which solvents were the most effective for extracting betulin and betulinic acid from Chinese white birch bark and found that the highest concentrations (20.2% of total mass for betulin and 1.86% for betulinic acid) were obtained when using 95% ethanol. In comparison, an acetone extraction suggested that the betulin content in the bark was 13.02%

and the betulinic acid content was 1.51%. These authors also found significant regional variations in the betulin and betulinic acid contents.

Liviu *et al*. (2012) analysed 10 birch bark samples collected from different locations in Romania and found that the betulin content ranged from 5.74% to 16.56% (with an average of 10.80%) and that the betulinic acid content ranged from 0.73% to 1.54% (with an average of 1.17%). Kim *et al*. (2013) collected birch bark samples in Korea and found that there was a seasonal variation in the betulin content, with it being higher in samples collected in the summer season.

2.2 Gallic Acid

Gallic acid (3,4,5 trihydroxy benzoic acid) is a secondary polyphenolic metabolite and is present in many plant species in the free form or as part of tannic acid molecule (Choubey, Varughese et al. 2015). Gallic acid is a common precursor of hydrolysable tannins (HTs). It transforms into complex galloylglucoses and ellagitannins via 1-O-galloylglucose (Ossipov, Salminen et al. 2003). Gallic acid and its esters are high in antioxidant activity and are also reported for their anti-carcinogenic, antimicrobial, anti-mutagenic, antiangiogenic and anti-inflammatory properties. They are also used in food preservation (Choubey, Varughese et al. 2015). Gallic acid has a reference price of $71/kg (http://www.molbase.com/en/cas-149-91-7.html?page=2).

Birch Leaves

Gallotanins concentrations, including the gallic acid concentration, in birch leaves were examined by Ossipov *et al.* (2003). The concentration of gallic acid in the leaves ranged from 0.245 mg/g to 0.050 mg/gdw. High concentrations of gallotannis were found in young leaves and the concentration decreased with an increase in leaf age. There was also significant variation in the individual gallotannin concentrations between the trees.

2.3 Catechin

Catechin, a plant secondary metabolite, is a flavan-3-ol, a type of natural phenol and antioxidant. (+)-Catechin and (-)-epicatechin are ubiquitous constituents of vascular plants, and frequent components of traditional herbal remedies, such as *Uncaria rhynchophylla*. They are also mostly found as cacao and tea constituents, as well as in Vitis vinifera grapes. Catechin and its derivatives are also reported in some wood species. In recent years, catechins are being used as natural antioxidant in oils and fats to protect against lipid oxidation and as supplements for animal feeds. Catechin is also used as an antimicrobial agent and functional ingredient in food and dietary supplements. The catechin wholesale price ranges from $100-200 per kg with the compound typically being extracted from tea. (https://www.alibaba.com/showroom/price-catechin.html).

Birch

The NORPYRO project in Finland investigated catechin concentrations in different parts of birch trees. Samples from three heights in a stem, the branch and the bark, were combined from

trees within the same geographical region. The samples were ground to 1 mm and extracted with acetone/water (95/5, v/v) with three successive extractions each lasting for 15 min. The concentrations of catechin, in the stem wood of *B.pendula* and *B.pubescens*, were in the range of 0.15 to 0.7 mg/g. In both species, the inner part of the stem wood had higher concentrations of catechins than the outer part of the stem wood. The concentrations of catechin xyloside were higher than the concentrations of catechin itself, whilst catechin-derived molecules such as gallocatechin 1 and 2 were present in minor concentrations (Roitto, Siwale et al. 2015).

Beech

Catechin is the most commonly reported flavonoid in beech wood. It is present in both catechin and the diastereomer form epi-catechin (Vek, Oven et al. 2016). The discoloration of beech wood is due to condensation of catechins to its polymers. Hence, extractable catechin concentration is reduced in discoloured beech woods due to its participation in formation of colour chromophores. Research on the hydrophilic extractives of discoloured and normal wood demonstrated that catechin is the dominant phenolic compound in beechwood. The content of catechin in the investigated beech trees ranged between 0.052 and 2.080 mg/g. It was also reported that catechin accumulated in wounded wood (Vek, Oven et al. 2013). Vek *et al.* (2014) reported catechin concentration of 0.8 mg/g in beech sapwood and less than 0.2 mg/g in red heart of beech by methanol extraction

Poplar

It has been reported that poplar trees synthesize flavan-3-ols (catechin and proanthocyanidins) as a defence against foliar rust fungi (Ullah, Tsai et al. 2019). The uninfected poplar nigra leaves have catechins concentrations at only 0.5 mg/g, however the leaves infected with rust fungi have increased catechin levels up to 3mg/g.

2.4 p-Coumaric Acid

p-Coumaric acid (p-CA), also known as 4-hydroxycinnamic acid, is a phenolic acid, which has been widely studied due to its potential pharmacological effects since it has high free radical scavenging, anti-inflammatory, antineoplastic, and antimicrobial activities, among other biological properties (Ferreira, Victorelli et al. 2019). The bulk price of 98% pure p-coumaric acid is $130- $150/kg. (https://www.alibaba.com/).

Wheat straw

Ferulic acid and p-coumaric acid are two major phenolics in wheat straw. Pan *et al.* (1998) reported 0.48% ferulic acid and 0.42% p-coumaric acid in wheat straw, with 56% of the ferulic acid and over 80% of the p-coumaric acid present in esterified form.

2.5 Acacetin

Acacetin (5,7-dihydroxy-4'-methoxyflavone) is a flavonoid compound with antimutagenic, antiplasmodial, antiperoxidant, anti-inflammatory and anticancer effects (Ri Kim, Gi Park et al. 2013). It also exhibits anti-inflammatory, anti-nociceptive, neuroprotective, and anti-

aromatase properties. It is a high-value chemical, selling for approximately $1500/kg (https://www.alibaba.com/)

Birch Foliage

Valkama *et al.* (2004) studied the changes in concentration of epicuticular flavonoids during leaf development in three Birch taxa. Leaves from short shoots were collected at six different stages of leaf development over a period of two months. From the results of that study, it was evident that acacetin concentration decreased with leaf age. In the case of *B. pubescens ssp. pubescens*, the acacetin concentration decreased from 6 mg/g to 3 mg/g with leaf growth from stage 1 to stage 6. In *B. pubescens ssp. Czerepanovii*, it decreased from 2.5 mg/g to 1 mg/g. In *B. pendula*, the concentration of acacetin in stage 1 young leaf was 0.8 mg/g and decreased to 0 at stage 6.

2.6 Chlorogenic Acid

Chlorogenic acids (CGAs) are a class of phytochemicals that are formed as esters between different derivatives of cinnamic acid and quinic acid molecules. In plants, accumulation of these compounds has been linked to several physiological responses against various stress factors (Ncube, Mhlongo et al. 2014). Chlorogenic acid is known for its antioxidant, antiinflammatroy and anti-canacer properties. It accounts for 5–10% of coffee beans, a much larger amount than caffeine (1–2%) (Izawa, Amino et al. 2010).

Beech Foliage

Pirvu *et al*. (2010) found, through ethanol extraction, a chlorogenic acid content of 43.35 mg/g in beech leaves. It has also been shown that the concentration of chlorogenic acid in beech leaves is dependent on the season; spring leaves reveal only some traces of chlorogenic acid, whilst summer to early-autumn leaves have shown the abundance of chlorogenic acid isomers, with these concentrations decreasing as the leaves progress to late-autumn (Pirvu 2013).

2.7 Salicilin

Salicilin is a well known compound, anti-inflammatory in nature, with pharamaceutical and cosmetic applications. It is a key component of constitutive resistance in trees against stem-boring insects in poplar trees and its concentration increases in bark and xylem during the infection (Wang, Qu et al. 2016). In poplar, the concentration of salicilin varies in different parts of plant. For example, bark from poplar twigs has 7 times more salicin than the bark of the tree trunk (Palo 1984).

2.8 Salicortin

Salicortin is an important component of the bark and leaves of all Populus and Salix species. Pearl and Darling (1969) reported 1.2% salicortin in birch bark with ethyl-acetate extraction.

2.9 Quercetin and Isoquercetin

Quercetin (3,3',4',5,7-pentahydroxyflavone), known for its antioxidant activity is one of the most widely-used bioflavonoids for the treatment of metabolic and inflammatory disorders (Anand David, Arulmoli et al. 2016). Quercetin is also reported for its anti-diabetic effect (Tanase, Coșarcă et al. 2019). Benedec *et al.* (2014) reported a quercetin concentration of 0.15 mg/g in *Populus tremula* leaves.

3 Analytical Techniques Undertaken

3.1 Sample Preparation and NIR Scanning

After the samples were received at the Celignis laboratory they were indexed and prepared for near infrared (NIR) analysis. The collection of NIR data is required in order to develop the predictive models targeted in Task 2.3 and Deliverable 2.6.

This analysis typically involved the following stages:

1. If the sample could be directly presented to the analytical cell of the NIR device, then direct NIR scanning of the samples as-received (i.e. wet and unground) was undertaken. Alternatively, the sample was chopped so that it could be placed inside the NIR cell and the spectra were then collected. These spectra were labelled WU and involved three cell packs (see below for description of packs) before averaging.
2. The sample was then left on a tray to air-dry in the Celignis laboratory for a number of days. Two sub-samples were also taken for as-received moisture content determination.
3. Once the sample was considered to be air-dry, it was scanned with the spectra labelled DU. This stage involved three cell packs before averaging.
4. The sample was then milled, typically in a multi-step process involving sieves of smaller apertures until the sample passed through a mill of 200 microns. The sample was then considered to be highly homogeneous in terms of its particle size distribution. It was then scanned with the spectra labelled DJ, and two cell packs were used to produce an averaged spectrum.
5. Samples in the DJ form were considered to be in a suitable state for chemical analysis.

For more information on the conditions used for the collection of the NIR spectral data please refer to UNRAVEL Deliverable D.2.2.

3.2 Ash Content

The ashing of a sample of known dry matter content involved using a Nabertherm L-240H1SN muffle furnace and a ramped heating program that attained a temperature of 575 ºC, which was maintained for 6 hours. This analysis was undertaken in duplicate for each sample.

3.3 Solvent Extraction of Samples

Four different types/combinations of solvents were used to remove the extractives from the milled and air-dried samples: water, acetone, 95% ethanol, and a sequential extraction involving water followed by 95% ethanol (termed "full-extraction"). Several Dionex Accelerated Solvent Extractor (ASE) 200 devices were employed for extractives removal. The protocol used is described below and was undertaken in duplicate for each sample:

1. A weighed amount of sample was introduced to an 11ml ASE extraction cell. The moisture content of the sample was also determined at the same time.
2. The cell was processed in the ASE 200 device using the chosen solvent, a pressure of 1500 PSI, a temperature of 100 ºC, a heat time of 5 minutes and a static cycle time of 7 minutes. Three static cycles were used for each sample and the total flush volume was 150%. The sample was then purged with nitrogen for 2 minutes, through the ASE cell, and the cell was then returned to the carousel of the ASE device.
3. The extract was collected in 60ml ASE collection tubes with the weight of extract recorded. A subsample of this extract was then stored for subsequent extractives analysis.
4. After extraction the, remaining solid was transferred to a box and left to air-dry for 2 days.
5. The weight of the sample in the box was then recorded along with the weight of the empty box. Subsamples were taken for moisture content determination at the same time as when the sample weight was measured.
6. Extractives were determined as the loss in dry matter associated with the extraction.
7. The remainder of the post-extraction solid residue was kept in sealed test-tubes for subsequent analysis, if required.

For full-extraction, the initial solvent used was water and, after step 3, the ASE cell was extracted again (step 2) using 95% ethanol, after which steps 3-7 were undertaken.

3.4 Analysis of Water Extracts for Carbohydrates

A 1ml subsample of the extract collected under water extraction was taken and 9ml of an internal standard solution (containing melibiose) was added. This diluted sample was then filtered through 0.2µm Teflon syringe filters, and put in vials for chromatographic analysis on a DIONEX ICS-3000 ion chromatography system comprising: an electrochemical detector (using Pulsed Amperometric Detection, PAD), a gradient pump, a temperature-controlled column and detector enclosure, and an AS50 autosampler. The AS50 injected 10 µl of the diluted sample, and sugar separation was achieved via the use of Carbo-Pac PA1 guard and analytical columns, connected in series. Sugar and sugar-alcohol separation (xylitol, mannitol, sorbitol, trehalose, arabinitol, arabinose, rhamnose, galactose, glucose, sucrose, xylose, mannose, and fructose, followed by the internal standard melibiose) occurred in 35 min with deionised water as the eluent, a flow rate of 1.5 mL/min, and a column/detector temperature of 17ºC. The standard Dionex "Carbohydrates" waveform was used for detection. PAD requires alakaline conditions for carbohydrate detection; hence NaOH (300 mM) was added to the post-column eluent stream, using a Dionex GP40 pump, at a flow rate of 0.3 mL/min.

After 35 min, a column regeneration step involving 400mM NaOH was undertaken for 4 minutes, preceded and followed by 2 minute gradients from/to water. There was a subsequent period of 5 minutes for equilibration, prior to the next injection, using only water as the eluent.

3.5 Analysis of Ethanol Extract

3.5.1 Sample Preparation

The contents of each ethanol-extraction collection vial were then transferred into pre-labelled 50 mL self-standing collection tubes and frozen. At a later point, a 4 mL aliquot from each of collection tube was taken and transferred into respective pre-labelled 15 mL tubes which were then placed in a TurboVap evaporator where they were fed with a constant steam of nitrogen (20 psi) for 20-30 minutes in order to remove the solvent. The weight of the empty tubes and the weight of the tubes containing the extractive solid residue after evaporation were recorded in order to establish the extractives mass balance.

The extractive solid residue was re-suspended into 1 mL methanol and the tube was vortexed for approximately 15 minutes in order to have a homogenous distribution in the solution. Finally, a 0.3 mL aliquot was taken from this solution and transferred into pre-labelled test vials, containing 1.2 mL of methanol for low concentration samples test vials and 0.15 mL of methanol for high concentration test vials. This process was repeated for both extractives replicates obtained after the extraction of each sample. Those test vials were used for the subsequent HPLC-DAD and LC-MS QTOF analysis of the extractives.

3.5.2 Preparation of Standards

According to the data found in the literature review, several standards were purchased from Sigma Aldrich to be used for the qualitative and quantitative analysis of compounds expected to be found in the samples. Stock standards were prepared by transferring approximately 5mg of each standard into different pre-labelled 1.5mL glass vials. Then approximately 1ml of methanol was added for standards to be used for HPLC analysis. The final concentration of most standards was 5mg/ml (except in a few instances where different concentrations were used due to practical issues in preparing the standards).

Finally, standards to be used for analysis were prepared by taking a 0.1mL aliquot from each stock standard and by diluting it with approximately 1.5mL of methanol for HPLC analysis.

3.5.3 Analysis of Ethanol Extracts using LC-MS QTOF

An Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system was used for the identification of analytes in the ethanol extracts. The conditions used for analysis are outlined below:

Instrument:	-	Agilent 6530 Accurate-Mass Q-TOF LC/MS
Column	-	InfinityLab Poroshell 120
Particle Size (µm)	-	2.7
Flow (mL/min)	-	0.7
Pressure (bar)	-	179
Injection Volume (µL)	-	2 & 5
Eluents	-	A = Deionized Water + 0.05% Formic Acid B = Acetonitrile + 0.05% Formic Acid
Gradient	-	0 min: 20% B 10 min: 50% B 15 min: 90% B 20 min: 90% B 26 min: 90% B 26.10 min: 20% B 30 min: 20% B
VWD Wavelenghts (nm)	-	205, 280
QTOF Tune Mass Range	-	Max = 3200 m/z Min = 100 m/z
Ionization Type	-	ESI
Q-TOF: Dual Gain Ratio	-	12
Q-TOF: MS Processing Mode	-	4
Q-TOF: Preamp Offset High Gain	-	-0.0879999995231628
Q-TOF: Preamp Offset Low Gain	-	50255

Standards were injected using 5 µl and 2 µl injection volumes in order to assess the linearity of the assay. During the analysis for quantification, a 2 µl injection volume was selected for certain samples due to the sensitivity of the instrument and the presence of too many compounds. For example, samples such as birch and beech barks were injected using 2 µl injections due to the high concentration of compounds present which made the qualitative and quantitative analysis difficult with a 5ul injection. In order to quantify the compounds, the peak areas from the VWD chromatograms at 280nm (for most compounds) and 205nm (for betulin and betulinic acid) were used.

Negative ionization was used for all the standards, except for betulin, due to the fact it did not ionize properly in negative ionization mode. Hence, positive ionization was used only for the betulin standard. Each sample was run twice, with the first run completed in negative ionization mode and the second run completed in positive ionization mode.

3.5.4 Analysis of Ethanol Extracts using HPLC-DAD-FLD

The following conditions for the HPLC-DAD-FLD analysis were established:

Instrument:	-	1260 Infinity II LC System
Column	-	InfinityLab Poroshell 120
Particle Size (µm)	-	2.7
Flow (mL/min)	-	0.7
Pressure (bar)	-	139
Injection Volume (µL)	-	5 & 20
Eluents	-	A = Deionized Water + 0.05% Formic Acid B = Acetonitrile + 0.05% Formic Acid

Gradient	-	0 min:	20% B
		10 min:	50% B
		15 min:	90% B
		20 min:	90% B
		26 min:	90% B
		26.10 min:	20% B
		30 min:	20% B
DAD Wavelenghts (nm)	-	205, 210, 280, 320, 340	

This system was used as a check on the performance of the Q-TOF system for quantification.

4 Results

4.1 Extractives Contents

Table 2 presents data for the extractives contents of the UNRAVEL samples. Data from four different types of extraction are presented (full, water, ethanol, and acetone). The extractives contents for each extraction were determined as the dry mass loss in the sample as a result of the extraction process. Below the results will be discussed for each feedstock type.

4.1.1 Birch

There is a wide variation in the amounts of different extractives present in the different birch fractions. The extractives contents are lowest in the birch chips (sample BIC-1-SAP) and greatest in the birch foliage (BIF-1-TNO) and birch bark (BIB-1-TNO). As would be expected, for all samples the full-extractives content is greater than either the water or ethanol extractives content due to the fact that the full extraction uses both of these solvents sequentially. However, the full extractives content is significantly less than the sum of the ethanol and water extractives contents, indicating that large amounts of the extractives are soluble in both water and 95% ethanol. The “Ethanol after Water” values in Table 2 are calculated by subtracting the water extractives content from the full extractives content and show the amount of ethanol-soluble extractives that are not soluble in water. The amount of ethanol soluble extractives present in each of the samples is somewhat similar to the amounts of water-soluble extractives, and, at relatively low extractive contents, the amount of acetone soluble extractives present are also similar. However, at higher water or ethanol extractive contents the amounts of acetone extractives tend to be lower (for example, the birch foliage has an ethanol extractives content of 16.46% and an acetone extractives content of 12.75%).

Given that stemwood chips (BIC-1-TNO and BIC-1-SAP) have significantly lower extractive contents than the bark sample (BIB-1-TNO), it would be expected that the sample containing both stem wood and bark (BIB-2-TNO) should have more extractives than the stem wood and less extractives than the bark sample, and this is indeed observed in the data. Data were not provided regarding the relative mass contributions that bark and stem wood contributed to sample BIB-2-TNO, however, using only the ethanol-extractives data, a bark contribution of 31.4% can be calculated (hence a stem wood contribution of 68.6%) based on the different amounts of extractives in BIC-1-TNO and BIB-1-TNO.

With regards to the relevance of these data to the FABIOLA fractionation process, the contents of acetone-soluble extractives are relatively low (1.9-2.5%) for the stem wood samples, but are significant for the foliage (12.8%) and bark samples (13.8%). Taking the example of the birch bark sample (BIB-1-TNO), prior lignocellulose analysis of the fully-extracted sample (as described in UNRAVEL deliverable D.2.2) found that it had a lignin content of 35.30% (with 34.10% Klason lignin and 1.20% acid-soluble lignin) and a hemicellulose content of 19.96% (calculated by subtracting the structural glucan content from the total structural sugars content). Hence, excluding consideration of the ash content, up to 69.08% of the sample may be expected to be solubilised in the FABIOLA fractionation (equal to the sum of the lignin, hemicellulose, and acetone extractives contents). The acetone extractives content of 13.82% would account for 20.01% of this solubilised fraction, a significant amount that would likely influence the efficient recovery and utilisation of the lignin and hemicellulose fractions. Hence, for birch, pre-extraction may well be warranted for bark or foliage feedstock or for mixed feedstocks containing significant quantities of either of these fractions. Given that the ethanol extractives contents of both the bark and foliage samples are greater than the corresponding acetone extractives contents, and given the fact that many compounds that can be extracted with acetone can also be extracted with ethanol, it is reasonable to assume that a pre-extraction using ethanol would remove much of the acetone extractives prior to the FABIOLA fractionation. Clearly, an acetone pre-extraction would also be effective in this regard. However, given that the water extractives represent 74% of the full-extractives content in the birch bark sample, a water extraction may also be effective in removing a sizeable component of the acetone-soluble compounds.

4.1.2 Beech

Many of the trends observed for the birch samples are repeated for the beech samples in Table 2. For example, the extractives contents of the stem wood chips are much lower than those of the bark and foliage. The branches have intermediate extractives contents and, based on the relative amounts of ethanol extractives in beech bark (BEB-1-TNO) and beech stem wood chips (BEC-1-TNO), a mass contribution from bark of 36.5% to the branches (POBR-1-TNO) can be calculated.

In general, the extractives contents for beech bark and foliage are less than for the corresponding fraction in birch, however an acetone extractives content of 9.15% for beech bark may still be high enough to warrant inclusion of a pre-extraction step prior to fractionation.

4.1.3 Poplar

The different fractions of poplar have higher extractive contents than the corresponding fractions for the birch and beech samples. For example, the full extractives content of poplar bark is 26.92% whilst for birch bark it is 19.45%. Interestingly, the stem wood chips (POC-1-TNO) have similar full-extractives contents to the beech branches (BEBR-1-TNO). No data were provided with regards to the age of the poplar tree(s) that were sampled, it is possible that this was a younger crop, which would be expected to have higher extractives contents. Additionally, for the poplar samples with higher extractives contents (e.g. bark, branch and foliage), the differences between the acetone extractives and ethanol extractives contents are more marked than for the birch and beech samples. For example, in birch branches (BIBR-1-

TNO) the acetone extractives content is 4.75% and the ethanol extractives content is 43.2% greater at 6.80% whilst for poplar branches (POBR-1-TNO) the acetone extractives content is 7.32% and the ethanol extractives content is 66.5% greater at 12.19%.

4.1.4 Olive Trees

Both olive residue samples (OTR-1-TNO and OTR-2-TNO) have similar profiles in terms of their extractive contents which are high (greater than 10% for all solvents used, excluding acetone). In these samples the differences between the acetone extractives contents and the ethanol extractives contents are even more marked than for the poplar samples (for example, the ethanol extractives content of sample OTR-1-TNO is 96.6% greater, in relative terms, than the acetone extractives content of that sample).

4.1.5 Grasses

Both grass samples (RSG-1-TNO (Road Side Grass) and SWG-1-TNO (Switchgrass)) have high contents of full, water, and ethanol extractives, although the acetone extractive content of the roadside grass sample is relatively low (2.96%), particularly when compared with its full extractives content (17.69%). In this particular instance, and in the context of obtaining a relatively clean (i.e. few extractives present) liquid fraction from FABIOLA pre-treatment, the pre-extraction of a high extractives content grass sample may be less important since only a small proportion of these extractives are soluble in acetone. However, if the target is obtaining a solid residue that is low in extractives content than a pre-extraction using water as a solvent would be warranted.

4.1.6 Straws

A total of five straw samples were analysed (wheat straw, rice straw, sunflower straw, rapeseed straw, and corn stover residues). These samples had somewhat similar extractives profiles which were notable for having significantly greater amounts of water extractives compared against ethanol or acetone extractives. For example, for rapeseed straw the ethanol extractives content was only 2.88% compared with a water extractives content of 10.56%. The differences between the water extractives and acetone extractives contents were even more marked.

4.1.7 Other Samples

The final two samples in Table 2 are CPH-1-TNO (cocoa pod husks) and CMF-1-TNO (cow manure fibres). These samples also have relatively high water and full extractives contents but significantly lower acetone extractives contents.

Table 2: Extractives contents of the UNRAVEL feedstock samples. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets.

Sample Type	Sample Name	Full Extractives (%)	Water Extractives (%)	Ethanol Extractives (%)	Ethanol after Water (%)	Acetone Extractives (%)
Birch	BIC-1-SAP - SAPPI Birch Chips Batch 1	2.66 (0.02)	1.77 (0.06)	1.65 (0.07)	0.89 (0.08)	1.87 (0.14)
	BIC-1-TNO - Birch Stemwood Chips	3.38 (0.18)	2.64 (0.05)	2.46 (0.15)	0.73 (0.22)	2.49 (0.20)
	BIB-1-TNO - Birch Bark	19.45 (0.13)	14.39 (0.83)	16.29 (1.01)	5.06 (0.70)	13.82 (0.18)
	BIB-2-TNO - Birch Wood with Bark	9.68 (0.35)	7.02 (0.09)	6.80 (0.16)	2.66 (0.26)	5.22 (0.22)
	BIBR-1-TNO - Birch Branches	9.99 (0.03)	6.24 (0.38)	6.80 (0.03)	3.75 (0.41)	4.75 (0.18)
	BIF-1-TNO - Birch Foliage	24.13	17.23	16.46 (0.23)	6.90	12.75 (0.40)
Beech	BEC-1-TNO - Beech Stemwood Chips	3.03 (0.46)	2.57 (0.19)	0.72 (0.43)	0.46 (0.27)	0.82 (0.28)
	BEB-1-TNO - Beech Bark	14.52 (0.69)	14.00 (0.01)	12.11 (0.02)	0.52 (0.68)	9.15 (0.09)
	BEBR-1-TNO - Beech Branches	7.83 (0.17)	7.02 (0.08)	4.88 (0.04)	0.81 (0.08)	3.41 (0.17)
	BEF-1-TNO - Beech Foliage	18.65 (0.01)	12.57 (0.07)	12.86 (1.20)	6.08 (0.06)	5.29 (0.28)
Poplar	POC-1-TNO - Poplar Stemwood Chips	7.06 (0.13)	3.06 (0.25)	3.77 (0.07)	4.00 (0.38)	3.28 (0.13)
	POB-1-TNO - Poplar Bark	26.92 (0.04)	18.45 (0.55)	20.74 (0.02)	8.46 (0.51)	15.30 (0.26)
	POBR-1-TNO - Poplar Branches	18.24 (0.87)	13.93 (0.86)	12.19 (0.01)	4.30 (0.01)	7.32 (0.04)
	POF-1-TNO - Poplar Foliage	30.47 (1.09)	23.76 (0.14)	20.70 (0.17)	6.71 (0.95)	14.17 (0.03)
Olive Trees	OTR-1-TNO - Olive Tree Residues	17.36 (0.06)	14.83 (0.25)	11.99 (0.05)	2.52 (0.30)	6.10 (0.28)
	OTR-2-TNO - Intact Olive Branches	16.52 (0.39)	16.21 (0.20)	12.73 (0.42)	0.32 (0.19)	7.50 (0.07)
Grasses	RSG-1-TNO - Road Side Grass	17.69 (0.17)	17.12 (0.04)	7.56 (0.61)	0.56 (0.14)	2.96 (0.17)
	SWG-1-TNO - Switchgrass	18.26 (0.03)	17.79 (0.09)	11.00 (0.24)	0.48 (0.12)	6.56 (0.03)
Straws	WHS-1-TNO - Wheat Straw	15.30 (0.21)	12.55 (0.36)	5.29 (0.36)	2.75 (0.57)	2.34 (0.33)
	RIS-1-TNO - Rice Straw	17.69 (0.62)	13.63 (0.07)	5.38 (0.59)	4.06 (0.55)	2.20 (0.23)
	SFS-1-TNO - Sunflower Straw	17.65 (0.18)	16.54 (0.05)	5.15 (0.17)	1.11 (0.13)	1.23 (0.24)
	RSS-1-TNO - Rapeseed Straw	10.56 (0.29)	10.12 (0.25)	2.88 (0.07)	0.43 (0.04)	1.78 (0.05)
	COS-1-TNO - Corn Stover Residues	16.85 (0.23)	15.06 (0.10)	6.21 (0.78)	1.79 (0.09)	3.81 (0.01)
Other	CPH-1-TNO - Cocoa Pod Husk Batch 1	22.56	19.54	10.10 (0.39)	3.02	5.96 (0.09)
	CMF-1-TNO - Cow Manure Fibres	12.09 (0.01)	7.95 (0.08)	3.56 (0.20)	4.14 (0.00)	2.41 (0.10)

4.2 Ash Contents after Full Extraction

Some ash may be removed in the extraction, depending on the composition of the ash and the types of solvents employed. The solid residues remaining after full extraction were retained and subsamples were ashed. The data obtained are presented in terms of the mass contribution to the original (non-extracted) sample in Table 3.

It can be seen that between 22% and 83% of the original ash is removed in full extraction. There are no clear trends in the data, however. For example, in birch bark and beech bark less than 25% of ash is removed in the full extraction, however in poplar bark 52% of the ash content is removed.

Table 3: Ash contents and the amount of ash retained in the biomass after full extraction. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets.

Sample Type	Sample Name	Original Ash Content (%)	Non-Soluble Ash (%)	Proportion of Ash Extracted (%)
Birch	BIC-1-SAP - SAPPI Birch Chips Batch 1	0.23 (0.03)	0.13 (0.04)	45.4%
	BIC-1-TNO - Birch Stemwood Chips	0.40 (0.02)	0.20 (0.01)	49.5%
	BIB-1-TNO - Birch Bark	2.26 (0.02)	1.76 (0.08)	22.1%
	BIB-2-TNO - Birch Wood with Bark	1.31 (0.03)	0.71 (0.04)	45.7%
	BIBR-1-TNO - Birch Branches	1.66 (0.02)	0.64 (0.01)	61.2%
Beech	BEC-1-TNO - Beech Stemwood Chips	0.57 (0.02)	0.10 (0.06)	83.2%
	BEB-1-TNO - Beech Bark	4.24 (0.00)	3.19 (0.07)	24.8%
	BEBR-1-TNO - Beech Branches	1.93 (0.05)	1.39 (0.25)	28.2%
	BEF-1-TNO - Beech Foliage	5.21 (0.01)	3.17 (0.01)	39.1%
Poplar	POC-1-TNO - Poplar Stemwood Chips	0.48 (0.00)	0.16 (0.04)	66.4%
	POB-1-TNO - Poplar Bark	3.04 (0.08)	1.46 (0.19)	52.0%
	POBR-1-TNO - Poplar Branches	2.25 (0.04)	1.19 (0.22)	47.0%
	POF-1-TNO - Poplar Foliage	5.21 (0.02)	2.54 (0.07)	51.3%
Olive Trees	OTR-1-TNO - Olive Tree Residues	1.26 (0.03)	0.72 (0.02)	42.6%
	OTR-2-TNO - Intact Olive Branches	1.26 (0.17)	0.95 (0.12)	24.7%
Grasses	RSG-1-TNO - Road Side Grass	12.44 (0.17)	5.79 (0.13)	53.5%
	SWG-1-TNO - Switchgrass	3.59 (0.02)	2.05 (0.16)	43.0%
Straws	WHS-1-TNO - Wheat Straw	7.55 (0.02)	4.13 (0.25)	45.3%
	RIS-1-TNO - Rice Straw	15.82 (0.03)	9.44 (0.20)	40.4%
	SFS-1-TNO - Sunflower Straw	9.86 (0.02)	1.79 (0.11)	81.8%
	RSS-1-TNO - Rapeseed Straw	6.22 (0.03)	2.00 (0.24)	67.9%
	COS-1-TNO - Corn Stover Residues	7.05 (0.13)	3.33 (0.09)	52.7%
Other	CMF-1-TNO - Cow Manure Fibres	12.51 (0.06)	7.92 (0.25)	36.7%

4.3 Carbohydrates in Water Extractives

Table 4 presents the total sugars, sucrose, glucose, fructose, and trehalose contents of the water extracts of the UNRAVEL samples. These data are presented in terms of the contribution these carbohydrates make to the total dry mass of the sample, for example 1.8% of the total mass of poplar foliage is water-soluble glucan (representing 7.6% of the total water extractives content of 23.8%). Table 5 presents the amounts of water-soluble mannose, galactose, rhamnose, xylose, and arabinose in the samples, whilst Table 6 presents the amounts of water soluble xylitol, sorbitol, and mannitol.

With regards to analysis of the lignocellulosic composition of a feedstock, it is important that these soluble sugars are removed prior to undertaking the analytical steps required to determine lignocellulose content. This is because, if these soluble sugars are not first removed, they could be incorrectly assumed to be coming from the structural polysaccharides of the sample.

Typically, the main carbohydrates in the water extract were glucose, sucrose, and fructose. For example, these three compounds contribute 96.8% to the total water-soluble sugars content for the switchgrass sample (SWG-1-TNO). However, for the two samples obtained from olive trees (OTR-1-TNO and OTR-2-TNO) trehalose was the main carbohydrate present in the water extract. Trehalose is a disaccharide that is formed by a 1,1-glycosidic bond between two α-glucose units. It is not typically found in large quantities in many plants, hence its presence at over 2% of total dry mass for the two olive tree samples was interesting.

There are no clear trends in the water-soluble carbohydrates data presented in Table 4 to Table 6. For instance, the total sugars content in birch bark (BIB-1-TNO) is greater than that of the birch stemwood chips (BIC-1-TNO). This would lead to an assumption (as with the ethanol and acetones extractives contents) that the amount of water-soluble carbohydrates in the sample of birch wood with bark (BIB-2-TNO) would be in between these two samples. However, that sample is seen to have a significantly higher total sugars content (1.46% versus 0.52% for the birch bark). Additionally, the total sugars content is high in switchgrass but low in the other grass sample (roadside grass, RSG-1-TNO). Water soluble sugars contents are somewhat similar in the straw samples, with the exception of the corn stover residues sample (COS-1-TNO) which contains a significant amount of water-soluble glucose (2.39%). The poplar samples have significantly higher water-soluble carbohydrates contents than the other hardwood samples, with the bark sample having the greatest sugars content.

It has been shown (Wulfes, Nyman et al. 1999) that the total amounts of water soluble carbohydrates and the relative proportions of the different soluble sugars tend to be much more dynamic in plants than the contents and proportions of the structural polysaccharides. This is becuase water-soluble-carbohdrates are short-lived intermediates and primary products of photosynthesis that serve functions in storage, translocation, and metabolic utilization of carbon as well as in protection against abiotic stresses. The needs for these array of functions vary greatly with growth cycle, nutritional state, tissue, season, and plant management practices and, as a result, it is hard to get be sure that the data presented for a single sample of each feedstock type (obtained under a specific set of conditions) will be reflective of that feedstock in different conditions. For example, it is possible that roadside grass may have much higher amounts of water-soluble carbohydrates if it were to be sampled from a different location or at a different time of the year.

Table 4: Results for the total sugars, sucrose, glucose, fructose and trehlose contents of the feedstock samples. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample.

Sample Type	Sample Name	Total Sugars (%)	Sucrose (%)	Glucose (%)	Fructose (%)	Trehalose (%)
Birch	BIC-1-SAP - SAPPI Birch Chips Batch 1	0.014 (0.001)				
	BIC-1-TNO - Birch Stemwood Chips	0.121 (0.080)	0.056 (0.036)	0.028 (0.018)	0.023 (0.015)	0.007 (0.005)
	BIB-1-TNO - Birch Bark	0.516 (0.015)	0.137 (0.005)	0.228 (0.003)	0.101 (0.008)	0.027 (0.000)
	BIB-2-TNO - Birch Wood with Bark	1.456 (0.014)	0.649 (0.002)	0.299 (0.007)	0.460 (0.004)	0.010 (0.000)
	BIBR-1-TNO - Birch Branches	0.167 (0.010)	0.035 (0.008)	0.061 (0.001)	0.012 (0.000)	0.028 (0.000)
Beech	BEC-1-TNO - Beech Stemwood Chips	0.044 (0.013)	0.004 (0.001)	0.010 (0.008)	0.006 (0.003)	0.009 (0.000)
	BEB-1-TNO - Beech Bark	0.415 (0.170)	0.057 (0.012)	0.137 (0.126)	0.120 (0.031)	
	BEBR-1-TNO - Beech Branches	0.684 (0.046)	0.102 (0.039)	0.264 (0.014)	0.215 (0.000)	0.045 (0.024)
	BEF-1-TNO - Beech Foliage	0.269 (0.025)		0.015 (0.003)		
Poplar	POC-1-TNO - Poplar Stemwood Chips	0.142 (0.007)	0.065 (0.000)	0.026 (0.001)	0.019 (0.001)	0.014 (0.003)
	POB-1-TNO - Poplar Bark	4.846 (0.092)	1.448 (0.645)	1.910 (0.408)	1.427 (0.327)	0.023 (0.000)
	POBR-1-TNO - Poplar Branches	1.431 (0.029)	0.402 (0.010)	0.500 (0.013)	0.337 (0.005)	0.039 (0.000)
	POF-1-TNO - Poplar Foliage	3.796 (0.307)	0.060 (0.002)	1.791 (0.216)	1.456 (0.043)	0.064 (0.008)
Olive Trees	OTR-1-TNO - Olive Tree Residues	4.257 (0.070)	0.653 (0.012)	0.609 (0.001)	0.486 (0.002)	2.409 (0.082)
	OTR-2-TNO - Intact Olive Branches	5.086 (0.022)	1.518 (0.081)	0.564 (0.041)		2.956 (0.058)
Grasses	RSG-1-TNO - Road Side Grass	0.066 (0.003)	0.015 (0.003)	0.002 (0.001)		
	SWG-1-TNO - Switchgrass	6.164 (0.051)	1.969 (0.570)	2.177 (0.365)	1.823 (0.220)	0.034 (0.003)
Straws	WHS-1-TNO - Wheat Straw	0.416 (0.028)		0.023 (0.001)		0.246 (0.044)
	RIS-1-TNO - Rice Straw	0.483 (0.001)		0.280 (0.040)	0.063 (0.035)	0.014 (0.002)
	SFS-1-TNO - Sunflower Straw	0.336 (0.027)		0.162 (0.006)	0.007 (0.001)	0.060 (0.003)
	RSS-1-TNO - Rapeseed Straw	0.367 (0.103)		0.177 (0.048)	0.029 (0.010)	0.047 (0.011)
	COS-1-TNO - Corn Stover Residues	4.788 (0.054)		2.387 (0.033)	0.812 (0.004)	0.202 (0.001)
Other	CMF-1-TNO - Cow Manure Fibres	0.055 (0.000)		0.008 (0.001)		0.001 (0.000)

Table 5: Results for the mannose, galactose, rhamnosae, xylose, and arabinose contents of the feedstock samples. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample.

Sample Type	Sample Name	Mannose (%)	Galactose (%)	Rhamnose (%)	Xylose (%)	Arabinose (%)
Birch	BIC-1-SAP - SAPPI Birch Chips Batch 1					
	BIC-1-TNO - Birch Stemwood Chips					0.002 (0.001)
	BIB-1-TNO - Birch Bark				0.007 (0.002)	0.005 (0.000)
	BIB-2-TNO - Birch Wood with Bark		0.018 (0.002)		0.003 (0.000)	0.002 (0.000)
	BIBR-1-TNO - Birch Branches	0.004 (0.001)	0.003 (0.000)	0.002 (0.000)	0.004 (0.000)	0.005 (0.000)
Beech	BEC-1-TNO - Beech Stemwood Chips		0.001 (0.000)		0.001 (0.000)	0.001 (0.000)
	BEB-1-TNO - Beech Bark		0.080 (0.001)	0.003 (0.001)		0.003 (0.000)
	BEBR-1-TNO - Beech Branches		0.009 (0.003)			0.003 (0.001)
	BEF-1-TNO - Beech Foliage		0.068 (0.006)	0.039 (0.005)	0.010 (0.003)	
Poplar	POC-1-TNO - Poplar Stemwood Chips					0.002 (0.001)
	POB-1-TNO - Poplar Bark	0.003 (0.001)	0.014 (0.002)			0.001 (0.000)
	POBR-1-TNO - Poplar Branches	0.010 (0.000)	0.033 (0.001)	0.034 (0.001)	0.006 (0.000)	0.035 (0.001)
	POF-1-TNO - Poplar Foliage	0.014 (0.001)	0.152 (0.015)	0.030 (0.002)	0.006 (0.000)	0.107 (0.021)
Olive Trees	OTR-1-TNO - Olive Tree Residues	0.005 (0.000)	0.022 (0.000)	0.049 (0.000)	0.005 (0.001)	0.009 (0.001)
	OTR-2-TNO - Intact Olive Branches		0.030 (0.003)	0.005 (0.000)		0.005 (0.001)
Grasses	RSG-1-TNO - Road Side Grass				0.004 (0.001)	0.002 (0.000)
	SWG-1-TNO - Switchgrass		0.082 (0.017)	0.040 (0.014)	0.010 (0.003)	0.013 (0.001)
Straws	WHS-1-TNO - Wheat Straw		0.022 (0.001)	0.002 (0.000)	0.057 (0.000)	0.001 (0.000)
	RIS-1-TNO - Rice Straw		0.049 (0.009)		0.029 (0.005)	0.026 (0.001)
	SFS-1-TNO - Sunflower Straw	0.044 (0.003)	0.006 (0.000)	0.002 (0.000)	0.004 (0.001)	0.002 (0.001)
	RSS-1-TNO - Rapeseed Straw	0.012 (0.004)	0.048 (0.015)		0.036 (0.010)	0.006 (0.001)
	COS-1-TNO - Corn Stover Residues		0.499 (0.028)		0.580 (0.010)	0.098 (0.002)
Other	CMF-1-TNO - Cow Manure Fibres				0.006 (0.001)	0.003 (0.000)

Table 6: Results for the xylitol, sorbitol, and mannitol contents of the feedstock samples. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample.

Sample Type	Sample Name	Xylitol (%)	Sorbitol (%)	Mannitol (%)
Birch	BIC-1-SAP - SAPPI Birch Chips Batch 1			0.013 (0.001)
	BIC-1-TNO - Birch Stemwood Chips			0.006 (0.005)
	BIB-1-TNO - Birch Bark		0.005 (0.000)	0.006 (0.000)
	BIB-2-TNO - Birch Wood with Bark		0.009 (0.002)	0.008 (0.000)
	BIBR-1-TNO - Birch Branches		0.001 (0.000)	0.010 (0.000)
Beech	BEC-1-TNO - Beech Stemwood Chips	0.002 (0.000)		0.011 (0.003)
	BEB-1-TNO - Beech Bark	0.009 (0.000)	0.007 (0.002)	
	BEBR-1-TNO - Beech Branches	0.030 (0.000)		0.016 (0.001)
	BEF-1-TNO - Beech Foliage	0.138 (0.020)		
Poplar	POC-1-TNO - Poplar Stemwood Chips			0.016 (0.000)
	POB-1-TNO - Poplar Bark		0.002 (0.000)	0.017 (0.001)
	POBR-1-TNO - Poplar Branches		0.013 (0.001)	0.022 (0.000)
	POF-1-TNO - Poplar Foliage	0.042 (0.000)	0.060 (0.001)	0.015 (0.002)
Olive Trees	OTR-1-TNO - Olive Tree Residues		0.010 (0.000)	
	OTR-2-TNO - Intact Olive Branches		0.009 (0.000)	
Grasses	RSG-1-TNO - Road Side Grass		0.041 (0.001)	0.002 (0.001)
	SWG-1-TNO - Switchgrass			0.016 (0.001)
Straws	WHS-1-TNO - Wheat Straw	0.001 (0.000)	0.014 (0.000)	0.049 (0.018)
	RIS-1-TNO - Rice Straw		0.004 (0.002)	0.017 (0.016)
	SFS-1-TNO - Sunflower Straw	0.003 (0.000)	0.008 (0.001)	0.039 (0.011)
	RSS-1-TNO - Rapeseed Straw		0.008 (0.002)	0.004 (0.003)
	COS-1-TNO - Corn Stover Residues		0.034 (0.000)	0.177 (0.014)
Other	CMF-1-TNO - Cow Manure Fibres			0.038 (0.001)

4.4 Data from the HPLC Analysis of the Ethanol Extract

Table 7 to Table 10 present the results for the HPLC quantitative analysis of a range of constituents in the ethanol extracts, using the analytical methods outlined in Section 3.5. These data are presented on the basis of the percent mass contribution (on a dry-solids basis) that these analytes contribute to the total mass balance of the sample. For all of the analytes in Table 7 (betulin, betulinic acid, catechin, gallic acid, procyanidin B2, catechin gallate, and p-Coumaric acid) their concentrations in the samples were determined using the corresponding standard (purchased from Sigma Aldrich) in HPLC analysis. For each of the analytes listed in Table 8 (theogallin, epicatechin gallate, acacetin, abrectorin, and methyl gallate), gallic acid was used as the standard for which to calculate the response factor to be used for the quantification of each analyte. For chlorogenic acid, oleuropein, and methyl oleuropein (Table 9) ferulic acid was used as the standard for quantification, whilst for isosalicin, salicortin (Table 9), quercetin, luteolin, and isoquercetin (Table 10) catechin was used. For aesculetin and aesculin (Table 10) p-coumaric acid was used as the standard for quantification.

Below the results of the HPLC analysis of the ethanol extracts will be discussed for each feedstock class.

Birch

Birch bark had by far the greatest concentration of the selected analytes, a result of its high betulinic acid (0.58%) and betulin (8.81%) contents. As discussed in Section 2.1, the concentrations of betulin and betulinic acid have been shown to vary considerably among birch bark samples, according to region, plant species, stage of grown, and plant parts (inner versus outer bark). For example, *birch pendula* is reported to have relatively low concentrations of betulin (4%), whilst the highest concentrations have been reported in Chinese white birch bark (20%). The type of extraction has been shown to play a key role in maximum extraction of betulin and its quantification, although the effect of solvent selection of betulinic acid has been shown to be less important. For example, Chinese white birch bark extracted with ethanol yielded 20% betulin and 1.86% betulinic acid, while acetone extraction yielded only 13% betulin and 1.51% betulinic acid (Zhao, Yan et al. 2007). As outlined in Section 3.3, the extractions undertaken in Task 2.1.2 of UNRAVEL involved the use of 95% v/v ethanol, using pressured liquid extraction at elevated temperatures over three extraction cycles. As a result, based on the literature review undertaken, it is expected that these conditions will result in the maximum amount of betulin present in the samples being removed during the extraction process. When undertaking experiments to optimise the pre-extraction conditions in Task 2.2.1 it will be important to determine, using these data as a baseline, the extraction efficiency of betulin and betulinic acid from the birch bark samples.

With regards to the other birch samples, betulin and betulinic acid were only detected in BIB-2-TNO (birch wood with bark). Since there was no betulin detected in the birch stemwood sample, in theory it should be possible to determine the bark content of sample BIB-2-TNO based on its betulin/betulinic-acid content and that of the birch bark sample. One the basis of the betulin content, the bark content of sample BIB-2-TNO was calculated to be 1.7% compared with a content of 10.21% using the betulinic acid content. In comparison, using only the ethanol extractives content as the basis for this calculation (as outlined in Section 4.1.1), the bark content was calculated as being 31.4%. Clearly, there are large differences between

these numbers, however there are a number of reasons why these differences may exist. Firstly, there was no clear detail on the specific origins of each of these samples, for example the birch bark sample may have been obtained from a different tree than BIB-2-TNO. Similarly, it was not clear from which part of the plant these samples were collected, for example the relative proportions between inner and outer bark.

In birch bark catechin was the third-most abundant compound quantified in the ethanol extract (0.138%). Unlike betulin and betulinic acid, catechin was not only detected in the bark sample but also in the stem wood chips (BIC-1-TNO) with a concentration of 0.010%. In contrast, the catechin content of the birch wood with bark sample (BIB-2-TNO) was 0.021%, implying a bark content of that sample (purely based on the catechin content) of 8.03%. Catechin was also detected in the birch branches sample (BIBR-1-TNO) at a slightly higher concentration (0.021%) than in the birch wood with bark sample.

The birch foliage sample (BIF-1-TNO) had a very different composition, with regard to the quantified analytes in the HPLC analysis of the ethanol extract, than the birch wood samples. For example, betulin, betulinic acid, and catechin were not detected but instead other analytes, not found to be present in the wood samples, were found. The most abundant analyte detected was acacetin (0.577%), followed by abrectorin (0.145%) and gallic acid (0.053%). The presence of acacetin was of particular interest, as the literature review undertaken showed that this compound is a high-value niche chemical (see Section 2.5). The determined concentration for acacetin is on the upper range of those found in the literature review, comparable to that found in early-stage leaves (Valkama, Salminen et al. 2004). Similarly, the concentration of gallic acid found in sample BIF-1-TNO was consistent with the level that may be expected in young leaves (as described in Section 2.2).

Beech

Amongst the different beech samples analysed, beech bark had by far greatest concentration of detected analytes (1.769% of total dry matter) in the HPLC analysis of the ethanol extract. This value was based on the detection of two analytes, catechin (1.240%) and proxyanidin B2 (0.529%). The detected catechin content in beech bark was significantly greater than that in the stem wood (0.006%) and branch (0.052%) samples. Unlike birch, no sample comprising wood and bark was supplied for analysis. Whilst catechin was detected at some level for all of the beech samples, procyanidin B2 was only detected in the beech bark sample.

With regard to the beech foliage sample, significantly less constituents were quantified in the HPLC analysis of the ethanol extract than for the birch foliage sample, with only catechin (0.027%) and chlorogenic acid (0.066%) found. This content of chlorogenic acid was among the lower range found in the literature and more consistent with early-season leaves (Pirvu 2013).

Poplar

The detected analytes in the HPLC analysis of the ethanol extracts of the poplar samples were significantly less than those in the extracts of the birch and beech samples. The constituent detected in the greatest quantities was quercetin (0.103% in the poplar branches sample, POBR-1-TNO). Salicortin was detected in the poplar bark sample (0.036%) and isosalicin (0.031%)

in the poplar foliage sample. In the poplar stemwood chips theogallin (0.066%) and epicatechin gallate (0.026%) were detected, however these analytes were not found in the poplar bark or branches samples.

Olive Tree Residues

Only low amounts of extractives were quantified in the HPLC analysis of the ethanol extracts of the olive tree residues. Oleuropein was found in sample OTR-1-TNO (0.012%). Oleuropein belongs to the secoiridoids, which are abundant in Oleaceae, Gentianaceae, Cornaleae, as well as many other plants. Oleuropein is typically found in the residues of the processing of olives (Termentzi, Halabalaki et al. 2015), however in the literature review no publications were found concerning the detection of oleuropein in the prunings of olive trees. Given that oleuropein was not detected in the other olive tree residue samples (OTR-2-TNO), it is possible that its presence in OTR-1-TNO was due to olive contamination of that sample. Sample OTR-1-TNO also had detectable levels of luteolin (0.010%), isoquercetin (0.043%), aesculetin (0.010%), and p-coumaric acid (0.002%) while these constituents were not detected in sample OTR-2-TNO. Luteolin has been detected in fresh olive pomace at concentrations of 0.035% (J.G. Sinrod, Avena-Bustillos et al. 2019) but no data were found in the literature for its concentration in olive tree wood. Similarly, p-coumaric acid has been shown to be present in olive oil residues (Lesage-Meessen, Navarro et al. 2001) but no data were found in the literature for its content in olive tree wood. These results add credence to the hypothesis that sample OTR-1-TNO has contamination of olives, olive-oil or of some residues of these rather than purely consisting of the wood prunings of the olive tree. Whilst those analytes associated with olives were not detected in sample OTR-2-TNO, aesculin was detected in that sample (0.082%) but not in OTR-1-TNO. Aesculin is a coumarin glucoside that naturally occurs in the trees horse chestnut, California buckeye, and prickly box, and in daphnin, as well as being found in dandelion coffee. A literature review was undertaken to find data concerning the content of aesculin in the prunings of olive trees but no relevant publications were found.

Road Side Grass

In the road side grass sample (RSG-1-TNO) only p-coumaric acid was detected, among the list of analytes presented in Table 7 to Table 10, in the ethanol extracts of the sample, being present at a concentration of 0.007%.

Wheat Straw

Similarly, only a small concentration of the sample was quantified from the HPLC analysis of the ethanol extract. Methyl oleuropein was found (0.014%) followed by p-coumaric acid (0.013%).

Table 7: Data for the concentrations of a range of constituents, expressed in their percent contribution to the total dry mass of the sample, found in the HPLC analysis of the ethanol extracts. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample.

Sample Type	Sample Name	Betulin (%)	Betulinic Acid (%)	Catechin (%)	Gallic Acid (%)	Procyanidin B2 (%)	Catechin Gallate (%)	p-Coumaric Acid (%)
Birch	BIB-1-TNO - Birch Bark	8.805 (0.205)	0.578 (0.010)	0.138 (0.009)				
	BIF-1-TNO - Birch Foliage				0.053 (0.001)			
	BIC-1-TNO - Birch Stemwood Chips			0.010 (0.000)				
	BIBR-1-TNO - Birch Branches			0.018 (0.001)				
	BIB-2-TNO - Birch Wood with Bark	0.150 (0.019)	0.059 (0.008)	0.021 (0.002)				
Beech	BEB-1-TNO - Beech Bark			1.240 (0.172)		0.529 (0.143)		
	BEF-1-TNO - Beech Foliage			0.027 (0.004)				
	BEC-1-TNO - Beech Stemwood Chips			0.006 (0.001)				
	BEBR-1-TNO - Beech Branches			0.052 (0.003)				
Poplar	POB-1-TNO - Poplar Bark							
	POF-1-TNO - Poplar Foliage						0.243 (0.031)	
	POC-1-TNO - Poplar Stemwood Chips							
	POBR-1-TNO - Poplar Branches							
Olive Trees	OTR-1-TNO - Olive Tree Residues							0.002 (0.004)
	OTR-2-TNO - Intact Olive Branches							
Grasses	RSG-1-TNO - Road Side Grass							0.007 (0.001)
Straws	WHS-1-TNO - Wheat Straw							0.013 (0.004)

Table 8: Data for the concentrations of theogallin, epicatechin gallate, acacetin, abrectorin, and methyl gallate, expressed in their percent contribution to the total dry mass of the sample, found in the HPLC analysis of the ethanol extracts. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample.

Sample Type	Sample Name	Theogallin (%)	Epicatechin Gallate (%)	Acacetin (%)	Abrectorin (%)	Methyl Gallate (%)
Birch	BIB-1-TNO - Birch Bark					
	BIF-1-TNO - Birch Foliage			0.577 (0.134)	0.145 (0.034)	
	BIC-1-TNO - Birch Stemwood Chips					0.024 (0.002)
	BIBR-1-TNO - Birch Branches					
	BIB-2-TNO - Birch Wood with Bark					
Beech	BEB-1-TNO - Beech Bark					
	BEF-1-TNO - Beech Foliage					
	BEC-1-TNO - Beech Stemwood Chips					
	BEBR-1-TNO - Beech Branches					
Poplar	POB-1-TNO - Poplar Bark					
	POF-1-TNO - Poplar Foliage					
	POC-1-TNO - Poplar Stemwood Chips	0.066 (0.002)	0.026 (0.001)			
	POBR-1-TNO - Poplar Branches					
Olive Trees	OTR-1-TNO - Olive Tree Residues					
	OTR-2-TNO - Intact Olive Branches					
Grasses	RSG-1-TNO - Road Side Grass					
Straws	WHS-1-TNO - Wheat Straw					

Table 9: Data for the concentrations of chlorogenic acid, oleuropein, methyl oleuropein, isosalicin, and salicortin, expressed in their percent contribution to the total dry mass of the sample, found in the HPLC analysis of the ethanol extracts. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample

Sample Type	Sample Name	Chlorogenic Acid (%)	Oleuropein (%)	Methyl Oleuropein (%)	Isosalicin (%)	Salicortin (%)
Birch	BIB-1-TNO - Birch Bark					
	BIF-1-TNO - Birch Foliage					
	BIC-1-TNO - Birch Stemwood Chips					
	BIBR-1-TNO - Birch Branches					
	BIB-2-TNO - Birch Wood with Bark					
Beech	BEB-1-TNO - Beech Bark					
	BEF-1-TNO - Beech Foliage	0.066 (0.004)				
	BEC-1-TNO - Beech Stemwood Chips		0.005 (0.000)			
	BEBR-1-TNO - Beech Branches					
Poplar	POB-1-TNO - Poplar Bark					0.036 (0.006)
	POF-1-TNO - Poplar Foliage				0.031 (0.005)	
	POC-1-TNO - Poplar Stemwood Chips					
	POBR-1-TNO - Poplar Branches					
Olive Trees	OTR-1-TNO - Olive Tree Residues		0.012 (0.001)			
	OTR-2-TNO - Intact Olive Branches					
Grasses	RSG-1-TNO - Road Side Grass					
Straws	WHS-1-TNO - Wheat Straw			0.014 (0.001)		

Table 10: Data for the concentrations of quercetin, luteolin, isoquercetin, aesculetin, and aesculin, expressed in their percent contribution to the total dry mass of the sample, found in the HPLC analysis of the ethanol extracts. The data presented are the average of two replicate analyses, with the standard deviation between the two analyses shown in brackets. If no numbers are present this indicates that the analyte was not detected in the sample

Sample Type	Sample Name	Quercetin (%)	Luteolin (%)	Isoquercetin (%)	Aesculetin (%)	Aesculin (%)
Birch	BIB-1-TNO - Birch Bark					
	BIF-1-TNO - Birch Foliage					
	BIC-1-TNO - Birch Stemwood Chips					
	BIBR-1-TNO - Birch Branches					
	BIB-2-TNO - Birch Wood with Bark					
Beech	BEB-1-TNO - Beech Bark					
	BEF-1-TNO - Beech Foliage					
	BEC-1-TNO - Beech Stemwood Chips					
	BEBR-1-TNO - Beech Branches					
Poplar	POB-1-TNO - Poplar Bark					
	POF-1-TNO - Poplar Foliage		0.010 (0.002)			
	POC-1-TNO - Poplar Stemwood Chips					
	POBR-1-TNO - Poplar Branches	0.103 (0.007)				
Olive Trees	OTR-1-TNO - Olive Tree Residues		0.010 (0.001)	0.043 (0.006)	0.010 (0.001)	
	OTR-2-TNO - Intact Olive Branches					0.082 (0.003)
Grasses	RSG-1-TNO - Road Side Grass					
Straws	WHS-1-TNO - Wheat Straw					

5 Conclusion

The extractives contents have been determined for a range of samples of relevance to the UNRAVEL process and the consortium partners for two main reasons: (1) to determine if these extractives may have an impact on the yield and quality of products in the fractionation process, and; (2) to determine if any specific extractive components are present in significant quantities to warrant focusing on these in Task 2.2.2 for increasing their purity in the pre-extraction. With regards to the first criterion, the influence of extractives on the efficacy of pre-treatment is not fully understood, however there is a general understanding that the greater the extractives contents the more influence these will have on process yields and product purities. For the FABIOLA fractionation that is at the core of the UNRAVEL process, the amount of acetone extractives is of particular importance as FABIOLA involves the use of acetone in fractionation. It was noted that the contents of acetone extractives were always lower than the contents of ethanol-soluble extractives in the same sample, but that, in many samples, the amounts of acetone soluble extractives were still significant.

With regards to the samples from hardwoods, it was noted that the extractives contents were typically least in the stem wood chips and greatest in the bark and foliage. The branch and non-debarked chip samples had intermediate contents of extractives, reflecting the contribution that bark makes to the total mass of those samples. It is the opinion of the Author that the extractives contents in the stem wood samples are sufficiently low that pre-extraction would not be warranted prior to the FABIOLA fractionation but that, in the bark, branches, and foliage samples, pre-extraction is likely to be necessary in order to improve the yield and quality of the components obtained in fractionation.

For the herbaceous samples (grasses and straws) the water extractives contents are typically significantly greater than the contents of extractives using organic solvents. At this stage of WP2 it is unclear with regards to the relative importance of water vs other extractives in their effects on fractionation, with these relationships to be explored later in the UNRAVEL project. However, prior work at ECN part of TNO on wheat straw suggested that pre-extraction was of benefit for FABIOLA fractionation.

With regards to the amounts of water-soluble sugars present in the samples, significant quantities were detected in some of the grass and straw samples (specifically the switchgrass and corn stover residues) as well as in the bark and foliage samples of the hardwoods and in the olive tree pruning samples. Of particular relevance was the relatively large amounts of sucrose and fructose present in a number of those samples. There exists the possibility that these constituents may be converted in the FABIOLA process to sugar degradation products such as hydroxymethyl furfural which may make biological conversion of the sugars more difficult. As a result, a water extraction may be necessary to remove these sugars. However, a part-water part-organic-solvent pre-extraction may be sufficient to remove these sugars as well as much of the other extractives, so simplifying the number of solvents and cycles needed in the pre-extraction and lowering the potential cost of this stage. This will be explored in UNRAVEL Task 2.2.1 where various types of partial extractions will be undertaken. Based on the results presented in this deliverable, the amounts of carbohydrates and other constituents present in those partial (simplified) extracts will be compared with the values obtained via full-

extraction in order to ascertain the efficacy of the partial extraction for the removal of specific constituents in the extractives.

With regards to the analysis of the extractives present in the organic extract of the biomass samples, a decision was made to focus on the analytes that, through the literature review, were expected to be present in greatest quantities and of most commercial relevance. Additionally, as determined in the literature review, these constituents were typically found to be highest in the ethanol extract so those extracts were focused on in order to calculate what the total concentrations of each relevant analyte were in each sample. The analysis of these extracts was undertaken under the context of deciding which analytes would be suitable to focus on in UNRAVEL Task 2.2.2 (“Increased Purity of Target Extractive Compounds”). The decision would be made on the basis of the concentration of the relevant analyte and its potential value and markets. It is the conclusion of the Author that only betulin and betulinic acid are present in sufficient quantities to warrant their inclusion in Task 2.2.2 and only for birch bark samples. The concentrations of these analytes in pre-extraction will be investigated both in Task 2.2.1 (“Development of a Simplified Extraction Scheme”) and in Task 2.2.2 with a target that maximal quantities (considering process costs) are extracted in Task 2.2.1 and maximal purities (again considering process costs) are obtained in Task 2.2.2.

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