3rd STSM – European Non-Wood Forest Products network

COST-STSM-FP1203-25353

Final Report

STSM Title: Chemical composition of polar bark extracts from different tree species

1. Purpose of the STSM:

1.1. State-of-the-art: Polyphenols

Knowledge of the chemical constituents of plants is helpful in the discovery of therapeutic agent as well as new sources of economic materials like new chemicals, oil and gums, and understanding the biosynthetic pathways and metabolism of some relevant compounds that protect plants from external aggressive agents as UV light, oxidants and biological microorganisms.[1]

Polyphenols are molecules from the plant kingdom that represent a wide range of substances with various structures.[1,2] The basic structure is composed of a benzene ring linked to one or more hydroxyl group, free or involved in another chemical function (e.g. ester, sugar). Polyphenols are aromatic compounds formed from the metabolism of shikimic acid and/or that of a polyacetyl.[1] Structurally, they fall into different families including anthocyanins, coumarins, tannins, lignins, flavonoids, quinones, acids and phenols (Figure 1).

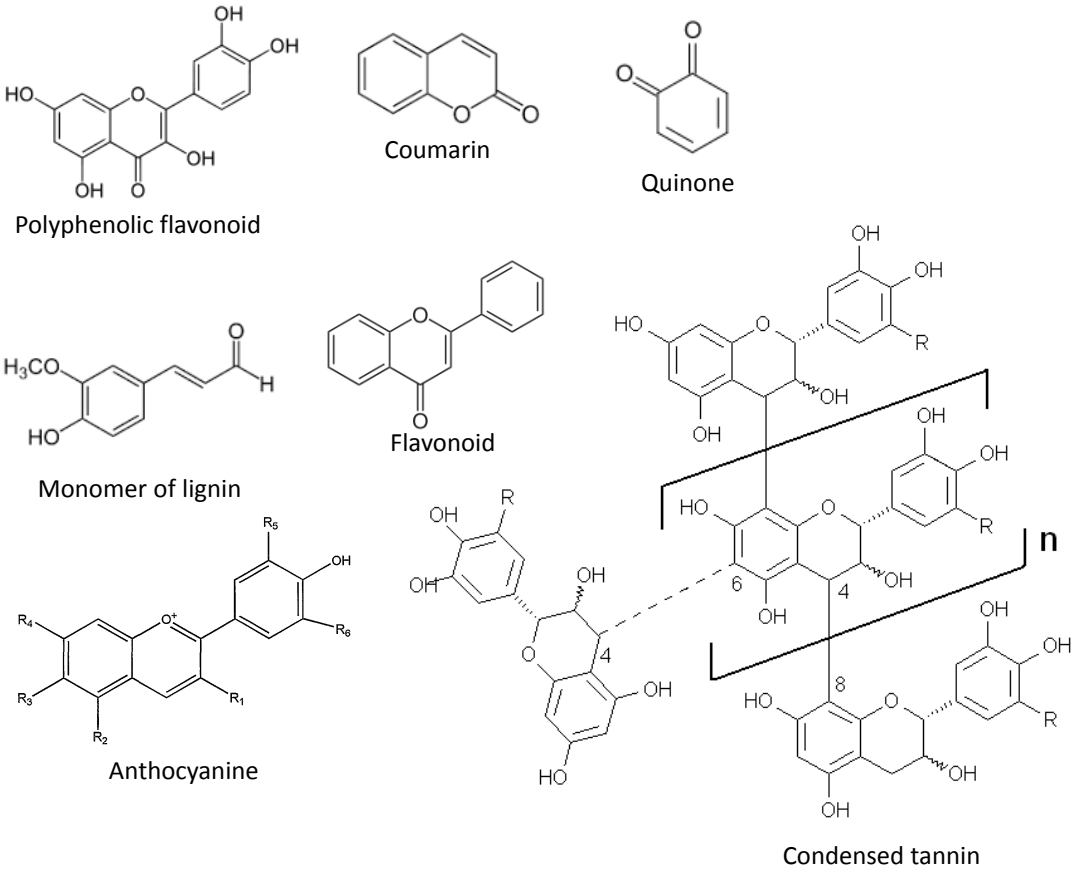


Figure 1 – Examples of different families of polyphenols

Plant polyphenols have been largely studied mainly because they might underlie the protective effects afforded by fruit and vegetable intake against chronic diseases.[3] Recently, there has been a surge in research on the potential role of antioxidants in the treatment of atherosclerosis,[4] heart failure,[5,6] liver dysfunction,[7, 8a,b] neurodegenerative disorders, cancer,[9] and diabetes mellitus.[10]

1.2. Chromatographic separation and identification techniques

1.2.1. High Performance Liquid Chromatography

Liquid chromatography is the preferred technique for both separation and quantification of phenolic compounds.[11] Various factors can have an effect on the analysis of phenolic by HPLC, namely sample purification, mobile phase, column types and detectors. In general, purified phenolics are applied to an HPLC system using a reverse phase C18 column (RP-C18), photo diode array detector (PDA) and polar acidified organic solvents.[12] Normally, HPLC sensitivity and detection is based on

purification of phenolics and pre-concentration from complex matrices of crude plant extracts.

The purification stage includes removing all the interfering compounds from the crude extract with partition able solvents and using an open column chromatography or and adsorption/desorption process.[13]

In terms of solvents, the most used are methanol and acetonitrile, or their aqueous forms.[13-15] The pH of the mobile phase has to be keep in the range of 2-4 to avoid the ionization of phenolics during separation and identification. Therefore, the aqueous mobile phases are acidified mainly with formic acid, acetic acid, phosphoric acid among others.[16] Also, a gradient elution system is more commonly applied than an isocratic elution system.[17]

One of the major concerns in identifying phenolics is the appropriate column selection. Generally, based on the polarity, different classes of phenolics can be detected using a normal phase C18 or reversed phase (RP-C18) column 10–30 cm in length, 3.9–4.6 mm ID and 3–10 μm particle size [18]. However, new types of columns (monolithic and superficially porous particles columns) from 3–25 cm length, 1–4.6 mm ID and 1.7–10 μm particle size are employed in phenolic detection by advanced HPLC techniques like UHPLC (ultra-high pressure chromatography) and HTLC (high temperature liquid chromatography) and two-dimensional liquid chromatography (LC × LC) [19,20]. Most HPLC assays of phenolics are carried out at ambient column temperature. Recently, however, higher temperatures have also been recommended due to new columns and instrumentation.[21,22] HPLC running time is the other factor that influences the detection of phenolics and can range from 10 to 150 min. Roggero et al. [23] emphasized that high reproducibility of results when long analysis times are employed requires constant temperature.

Diode array detectors (also referred to as a DAD detector or more specifically HPLC PDA detector) are used for obtaining spectral profiles from molecular mixtures or chromatographically separated samples. Phenolics are often identified using UV-Vis and photodiode array (PDA) detectors at wavelengths between 190-380 nm [24, 25]. However, HPLC coupled with MS detectors is a highly sensitive and has the power to achieve high specificity due to the mass selectivity of detection [26]. HPLC–NMR and UHPLC are the other novel techniques to identify bioactive compounds in new sources of rare natural products [27, 28]

1.2.2. Other assays used for separation and quantification of phenolics

Thin-layer chromatography (TLC) is a partitioning technique employed to separate phenolics in foods [29] and is a powerful technique to analyze phenolics, especially in crude plant extracts. Phenolics in crude plant extracts can be separated by a number of TLC techniques, which are cheap and provide multiple detection on the same TLC plate in a short analysis time [29]. Sajewicz et al. [30] indicated that a silica gel TLC-based video imaging method is a valuable complementary fingerprint technique to identify phenolic acids and flavonoids fractions from different sage species. de Oliveira et al. [31] also utilized silica gel TLC to identify phenolic compounds from *Baccharis trimera* extract.

The biology and health benefits of phenolics have lead researchers to discover, modify and utilize techniques for the extraction, separation and quantification of these compounds from natural sources. These methods need to be simple, rapid, environmentally friendly and comprehensive.

1.3. Objectives

The main objectives proposed for the present STSM were:

1 – Phytochemical analysis by HPLC in order to identify qualitatively the polar compounds present in methanolic extracts from different bark tree species namely *Pseudotsuga menziesii*, *Betula pendula*, *Quercus suber, Quercus variabilis* and *Quercus cerris*.

2 – Comparison between the composition of polar ethanolic extracts from *Pseudotsuga menziesii*, *Betula pendula, Quercus suber, Quercus variabilis* e *Quercus cerris*.

3 – Evaluate the efficiency of the technique used HPLC-DAD for the identification of the polar compounds in the extracts mentioned above.

4 – Comparison between the two techniques HPLC-RI and HPLC-DAD used for the identification of the polar extractives present.

2. Description of the work carried out during the STSM

2.1. Bibliographic research

There are chemical characteristics of compounds that allow their separation by HPLC:

- polarity;
- electrical charge;
- molecular size.

The structure, activity and physical-chemical properties of a certain molecule are determined not only by the arrangement of the constituent atoms (functional groups) but also the bonds between them, determining if the molecule is polar or non-polar. Organic molecules are sorted into classes according to the principal functional group(s) that each contains. Using a separation mode based on polarity, the relative chromatographic retention of different kinds of molecules is largely determined by the nature and location of these functional groups.[32]

Molecules with similar chromatographic polarity tend to be attracted to each other; those with dissimilar polarity exhibit much weaker attraction, if any, and may even repel one another. This becomes the basis for chromatographic separation modes based on polarity.

To design a chromatographic separation system [Fig.2], we create competition for the various compounds contained in the sample by choosing a mobile phase and a stationary phase with different polarities. Then, compounds in the sample that are similar in polarity to the stationary phase [column packing material] will be delayed because they are more strongly attracted to the particles. Compounds whose polarity is similar to that of the mobile phase will be preferentially attracted to it and move faster.

Figure 2 –Representation of a chromatographic separation system

In this way, based upon differences in the relative attraction of each compound for each phase, a separation is created by changing the speeds of the analytes.

Normal-Phase HPLC

In his separations of plant extracts, Tswett was successful using a polar stationary phase with a much less polar [non-polar] mobile phase. This classical mode of chromatography became known as normal phase.[33]

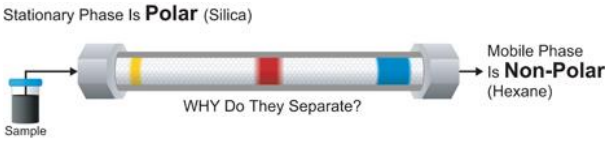


Figure 3 – Representation of a normal-phase HPLC

In the figure above (Fig. 3), the stationary phase is polar and retains the polar yellow dye most strongly. The relatively non-polar blue dye is won in the retention competition by the mobile phase, a non-polar solvent, and elutes quickly. Since the blue dye is most like the mobile phase [both are non-polar], it moves faster. It is typical for normal-phase chromatography on silica that the mobile phase is 100% organic; no water is used.

Reversed-Phase HPLC

The term reversed-phase describes the chromatography mode that is just the opposite of normal phase, namely the use of a polar mobile phase and a non-polar [hydrophobic] stationary phase (Fig. 4).

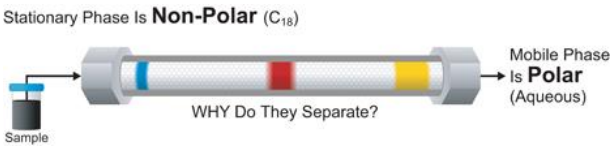


Figure 4 - Representation of a normal-phase HPLC

Now the most strongly retained compound is the more non-polar blue dye, as its attraction to the non-polar stationary phase is greatest. The polar yellow dye, being weakly retained, is won in competition by the polar, aqueous mobile phase, moves the fastest through the bed, and elutes earliest.[32,33]

Today, because it is more reproducible and has broad applicability, reversed-phase chromatography is used for approximately 75% of all HPLC methods. Most of these protocols use as the mobile phase an aqueous blend of water with a miscible, polar

organic solvent, such as acetonitrile or methanol. This typically ensures the proper interaction of analytes with the non-polar, hydrophobic particle surface. A C18–bonded silica [sometimes called ODS] is the most popular type of reversed-phase HPLC packing.[33]

2.2. Preparation of ethanolic extracts from barks

2.2.1. Samples

The bark samples from *Pseudotsuga menziesii*, *Betula pendula*, *Quercus cerris*, *Quercus suber* and *Quercus variabilis* were collected from different locations and stored in indoor conditions with low light and good ventilation. From *Pseudotsuga menziesii* and *Quercus variabilis* we were also able of separate the visible layers of cork.

2.2.2. Fractioning

After air-drying at ambient conditions, the barks from each species were mixed into an homogenized sample and ground in a cutting mill (Retsch SM 2000) using an output sieve with 10 mmm x 10 mm openings, and sieved with a vibratory sieving apparatus (Retsch AS 200 basic) with standard sieves with the following mesh sizes: 80 (0.180 mm), 60 (0.250 mm), 40 (0.425 mm), 20 (0.850 mm), 15 (1.0 mm) and 10 (2.0 mm). After sieving, the mass retained on each sieve was weighed and the corresponding mass fraction yields were determined.

The general chemical composition included determination of ash, extractives soluble in dichloromethane, methanol and water, suberin, lignin and polysaccharides. The extraction with organic solvents and water was performed in a Soxhlet apparatus during 6 h for the first extraction with dichloromethane and 16 h for ethanol and water extractions. Solvents were recovered and the extractives content determined from the mass of solid residue after drying at 100-105 ºC, and reported as mass percentage of the original samples.

The ethanol extracts were dried under nitrogen flow and a vacuum drying oven during 24 hours.

2.2.3. Total phenolic, flavonoid content and tannin content determination

The total phenolic content of the bark extracts in ethanol was determined using a modified Folin-Ciocalteu method [34], which is based on the reduction of a

phosphowolframate-phosphomolibdate complex by phenolics to blue reaction products. Gallic acid (GA) was used as standard.

The aluminum chloride method was used for the determination of the total flavonoid content of the bark extracts in ethanol and water.[35] Catechin (CA) was used as standard.

Condensed tannins content in ethanol extracts of bark was determined by the vannilin-sulphuric acid assay using catechin as standard.[36] Hydrolysable tannins content was determined with the tannic acid assay.[37]

2.3. Choosing the appropriate HPLC column for the extracts analysis

The HPLC-PDA system consisted of a ThermoScientific Acella Autosampler (AS), Acella Pump 600 (SN: 20312) and Acella PDA detector (Fig. 5). The system was operated under the ChromQuest 5.0 software.

Figure 5 – ThermoScientific Accella Autosampler, Accella Pump 600 and Accella PDA detector

For the qualitative analysis of the different bark ethanol extracts there were several columns tested for an efficient separation of the existing compounds.

Tested columns:

- ThermoScientific Accucore RP-C18 PFP
- ThermoScientific Hypersil ODS RP-C18
- ThermoScientific Accucore RP-C18

a) *ThermoScientific Accucore RP-C18 PFP*

The columns from Accucore RP-C18 PFP have a pentafluorophenyl (PFP) stationary phase (suitable for a wide range of compounds:polar, basic and hydrophobic) which provides a unique selectivity for halogenated species and nonhalogenated polar compounds, separate conformational and positional isomers.[38] These columns allow fast separations and its 2.6µm core-enhanced technology particles enable operation at high flow rates without generating excessive backpressures.[39]

Chromatograms have excellent peak shapes enhancing resolution and sensitivity, mostly because of their solid-core 2.6µm particles and narrow particle size distribution that reduces band broadening and improve separation efficiencies. Besides that, high surface coverage of silica minimizes secondary interactions and peak tailing.[39]

These columns allow excellent reproducibility and robustness.[39]

b) *ThermoScientific Hypersil ODS RP-C18*

Hypersil stationary phases are recognized as an industry standard in HPLC. Proven as an effective analytical tool, the classical Hypersil phases are well established and referenced in many HPLC methods worldwide.[39]

c) *ThermoScientific Accucore RP-C18*

This column allows an optimal selectivity for nonpolar compounds and is based on a hydrophobic interaction mechanism.[39]

Separations are fast and again, the 2.6µm core-enhanced technology particles enable operation at high flow rates without generating excessive backpressures. [39]

Chromatograms show excellent peak shapes for enhanced resolution and sensitivity because of the solid core 2.6µm particles and narrow particle size distribution that reduces band broadening and improve separation efficiencies. [39]

High surface coverage of silica minimizes secondary interactions and peak tailing. [39]

Again, these columns allow excellent reproducibility and robustness. [39]

Table 1 – Columns used to analyze the bark polar extracts samples

Columns	Length (mm)	Diameter (mm)	Particle Size (μm)
Hypersil ODS RP-C18	150	4.6	5
Accucore RP-C18 PFP	150	2.1	2.6
Accucore RP-C18	150	4.6	2.6

2.4. Preparation of samples for HPLC-PDA analysis

Samples of each bark ethanolic extract were first prepared as stock-solutions with a concentration of 0.1 mg ml^{-1} and stored in ambar flasks to protect them from possible light degradation. Samples were dissolved in an ultrasound system (5 minutes) and then filtered through 0.20 μm filters.

The samples (3 μl were injected) were analyzed using methanol (solvent A) and water (solvent B), both acidified with 0.1% formic acid as mobile phases, and with the chosen elution program at a flow rate of 0.4 ml min^{-1} (Program 1: 10 min 10% A, 80 min gradient until 100% A, isocratic for 20 min, gradient from 100% A until 10% in 0.5 min, restoring initial conditions and staying isocratic for 9.5 min) with the Accucore RP-C18 column.

After analysis of the obtained chromatograms we concluded that it was necessary to increase the samples concentration to 0.5 mg ml^{-1}, and they were prepared as mentioned above, but they were filtered through 0.45 μm filters to avoid retention of bigger molecules. The same elution gradient conditions were used (flow rate of flow of 0.4 ml min^{-1}, Program 1) increasing however the volume of sample injected to 5 μl.

After analysis, we chose the optimal concentration of bark samples (0.5 mg ml^{-1}), the appropriate filter dimensions (0.45 μm with 25 mm of diameter)

2.5. Choosing the appropriate solvents, gradient programs, columns for a correct separation and identification of compounds

Chemical separations can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase.

The extent or degree of separation is mostly determined by the choice of stationary phase (as mentioned above) and mobile phase [33,39]. Generally the identification and separation of phytochemicals can be accomplished using isocratic system (using single unchanging mobile phase system). Gradient elution in which the proportion of organic solvent to water is altered with time may be desirable if more than one sample component is being studied and differ from each other significantly in retention under the conditions employed.

The first condition tested included:

- Accucore RP-C18 column,
- MeOH+0.1% HCOOH (solvent A)
- H_2O+0.1% HCOOH (solvent B)
- and the elution system studied was based in an existing gradient elution program (Program 1) with 0.4 ml min^{-1} flow-rate:

Table 2 – Elution Program 1

Time (min)	A %	B%
0.00	10	90
10.00	10	90
90.00	100	0
110.00	100	0
110.50	10	90
120.00	10	90
	100	0

We started our study with *Betula pendula* ethanol extract and after finding the best concentration for the analysis (0.5 mg ml^{-1}) and the appropriate filter to be used (0.45 μm), the chromatogram obtained showed good resolution peaks and well separated.

So we proceed with the same conditions to the other ethanol bark samples. However, for the other bark samples from *Quercus suber, Q. cerris, Q. variabilis* and *P. menziesii* or even for cork and phloem portions of *P. menziesii*'s bark we could not have good chromatograms (no resolution, few peaks with very low intensity). In order to solve this problem, several conditions were changed and tested, namely using other type of columns, other elution system and other gradient programs.

We started to change the solvent used (Solvent A: acetonitrile + 0.1% HCOOH), however, for the other bark samples from *Quercus suber, Q. cerris, Q. variabilis* and *P. menziesii* or even for cork and phloem portions of *P. menziesii*'s bark we still could not have good chromatograms. The same happens when we used as mobile phases water/acetonitrile (90:10, v/v) (A) and acetonitrile (B), both with 0.1% of formic acid.[39] Santos et al. in which solvent A consisted on water:acetonitrile 90:10 + 0.1% HCOOH and as solvent B acetonitrile + 0.1% HCOOH. The following linear gradient was applied (Program 2): 0-3 min, 0% B; 3-10 min, 0-10% B; 10-30 min, 10-20% B; 30-35 min, 20-25% B; 35-50 min, 25-50% B; 50-60 min, 50-0% B.

After these changes we decided that we should keep the first elution program (Program 1) and the same mobile phases tested (Solvent A: MeOH + 0.1% HCOOH and solvent B: water + 0.1% HCOOH).

As we still could not see any peaks from the extracts of the species mentioned above, we tried also to change the columns and were tested an Accucore PFP and an ODS Hypersil. With the ODS Hypersil column we saw some small peaks in *Quercus* bark samples, but they were too small, and with no good resolution.

From the literature, we know that some complex samples need a pre-treatment before they can be analyse in HPLC. In order to do so, we decided to apply a liquid-liquid extraction in ethanol extracts from bark samples of *Quercus suber, Q. variabilis, Q. cerris* and *Pseudotsuga*, and separate them by polarity using cyclohexane to remove the partially non-polar compounds, dichloromethane and then ethyl acetate for the most polar compounds. These fractions from each bark extracts were dried under vacuum during 48 hours, and weight to determine the yield of each extraction. Stock solutions of each fraction with the optimized concentration of 0.5 mg ml^{-1} were prepared, redissolved in methanol and injected in the HPLC system with MeOH + 0.1% HCOOH and water + 0.1% HCOOH as mobile phases, and the elution program mentioned above as Program 1.

In these conditions, we were able to obtain chromatograms relatively well defined, with a good resolution and peaks relatively well separated for all the samples analyzed. However, there was the need of shortening the time of the run because the first 15-20 minutes did not show any peaks, in either of the fractions of each bark extracts.

In order to optimize the run, the elution program was modified (Table 3):

Table 3 – Elution Program 3

Time (min)	A (%)	B (%)
0.00	30	70
5.00	35	65
15.00	45	55
25.00	70	30
30.00	100	0
40.00	100	0
	100	0

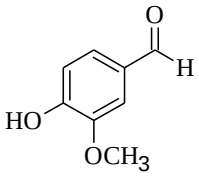
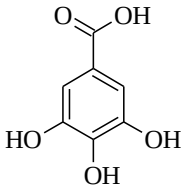
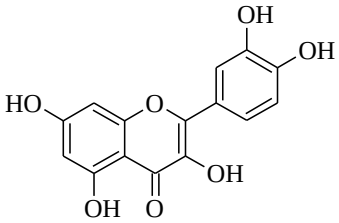
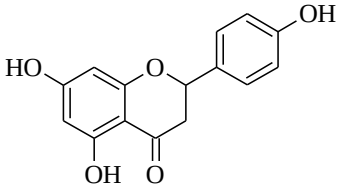
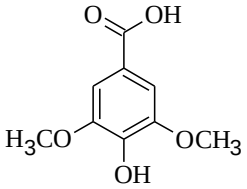
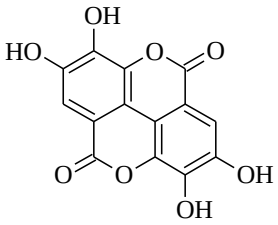
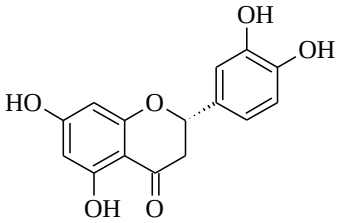
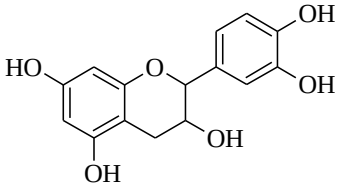
The selected elution program (Program 3) seems to be the most suitable for the separation of the different peaks that appear in the chromatograms of the fractions from the ethanol extracts of the bark species in study (and also cork and phloem from *P. menziesii*).

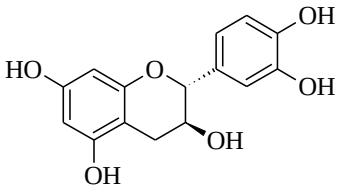
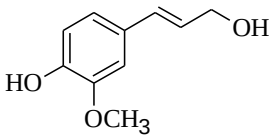
2.6. Choosing the right and available standards for the qualitative analysis of the extracts

Unfortunately it is impossible to have all the standards we need to identify all the peaks from the chromatograms, unless we already know which compounds exist in each sample already and have the financial availability to request all the standards we want to test on our samples.

From the literature we found some compounds already identified in similar species and also in *Q. suber* polar extracts; so according to the available standards, we choose for the qualitative analysis of our samples the following external standards:

Table 4 – Standards used for the analysis of the samples

Nº	Standard's name	Structure
1	Vanilin	
2	Gallic acid	
3	Quercetin	
4	Naringenin	
5	Syringic acid	
6	Ellagic acid	
7	Eriodictyol	
8	(-)-Epicatechin	

9	(+)-Catechin	
10	Coniferyl alcohol	

The stock solutions of these standards were prepared with 0.5 mg ml^{-1} concentration.

Each standard was injected individually and it was also prepared a solution containing all the standards in methanol in order to identify the retention time characteristic of each standard alone and in the presence of each other.

The compounds selected for standards appear in the following order, based on their retention times, with the selected mobile phases (A: MeOH + 0.1% HCOOH and B: water + 0.1% HCOOH) and elution program (Program 3).

Table 5 – Retention times for the standards used when injected individually, and in mixture

Standard	Rt (min) individual	Rt (min) in mixture
Gallic acid	5.775	5.817
(+) Catechin	18.313	18.372
(-) Epicatechin	22.740	22.765
Syringic acid	23.168	23.188
Vanilin	23.885	23.915
Conipheryl alcohol	24.977	24.980
Ellagic acid	30.057	30.170
Eriodictyol	32.400	32.413
Quercetin	34.120	34.175
Naringenin	34.850	34.848

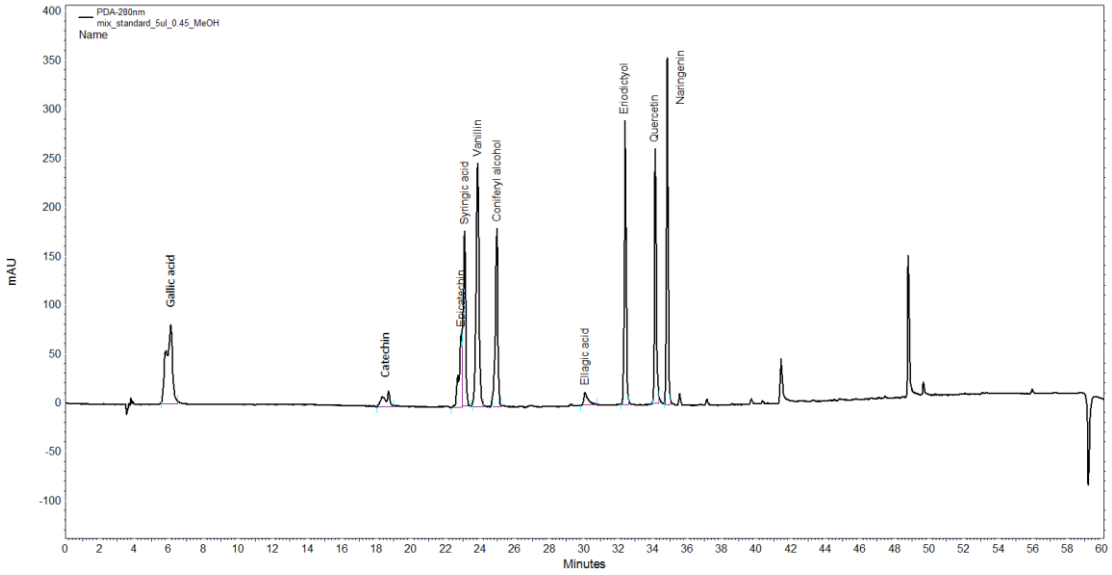


Figure 6 – Chromatogram of the mixture of the used external standards

It is worth of noticing that gallic acid appears as two peaks, which is due to the fact that an acid in the presence of an alcohol as methanol in an acidic medium (mobile phase) promotes the formation of an esterified structure (Fig. 7), meaning that first elutes the gallic acid and then the esterified structure.

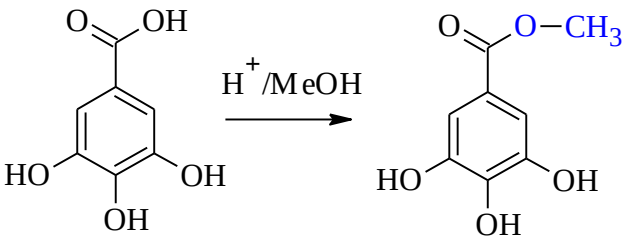


Figure 7 – Esterification of gallic acid

It was also a surprise that the peaks from (+)-catechin and (-)-epicatechin appear both with shoulder, probably due to isomerism.

2.7. Method validation

The validation of the method choose for the analysis of the ethanol bark samples needs to be validated, meaning that some characteristics were and are being tested and evaluated, namely: accuracy, repeatability, detection limit, quantitation limit, linearity and range.

Although the main objectives of this STSM only concerned the qualitative analysis of the extracts, we performed (and is still undergoing in collaboration with the host institution) a quantitative analysis for posterior publication of the results. So, for the criteria mentioned above, we already did the linearity and accuracy tests in which standard solutions with five different concentrations (0.5 mg ml^{-1}, 0.1 mg ml^{-1}, 0.05 mg ml^{-1}, 0.025 mg ml^{-1} and 0.0125 mg ml^{-1}). Tree individually replicates of each concentration were analyzed. The method of standard preparation and the number of injections were the same as used in the final procedure. The determination of acceptance criteria is undergoing.

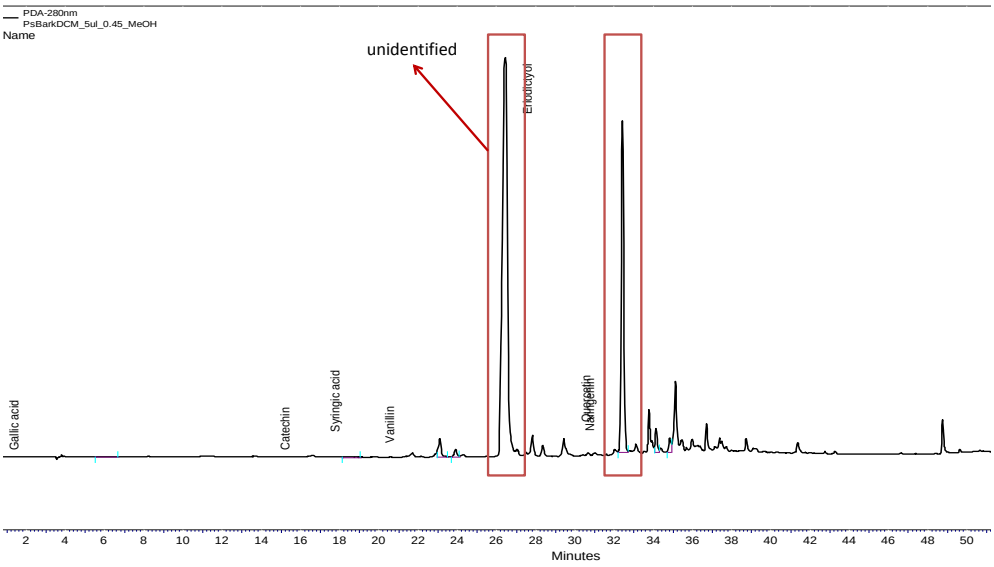
Determination of repeatability, limits of detection and quantification are undergoing.

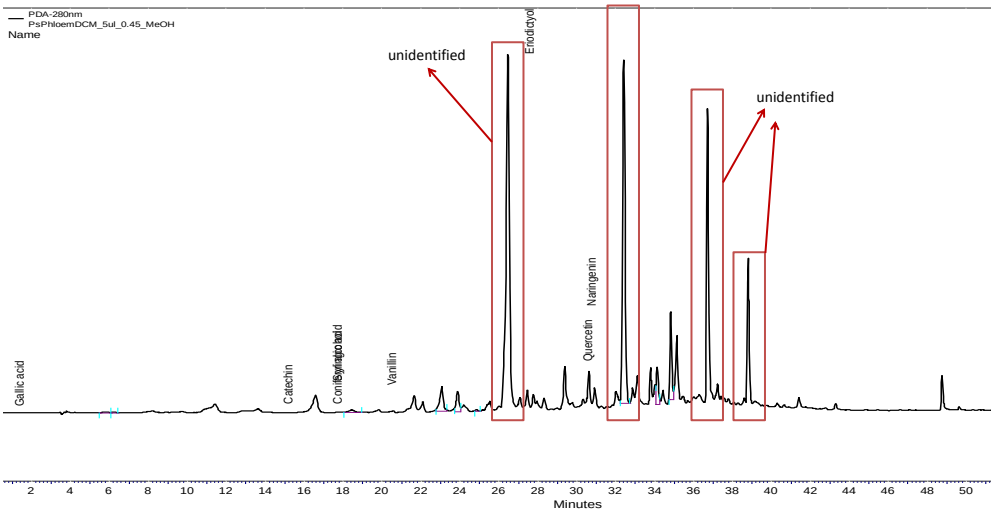
3. Main conclusions

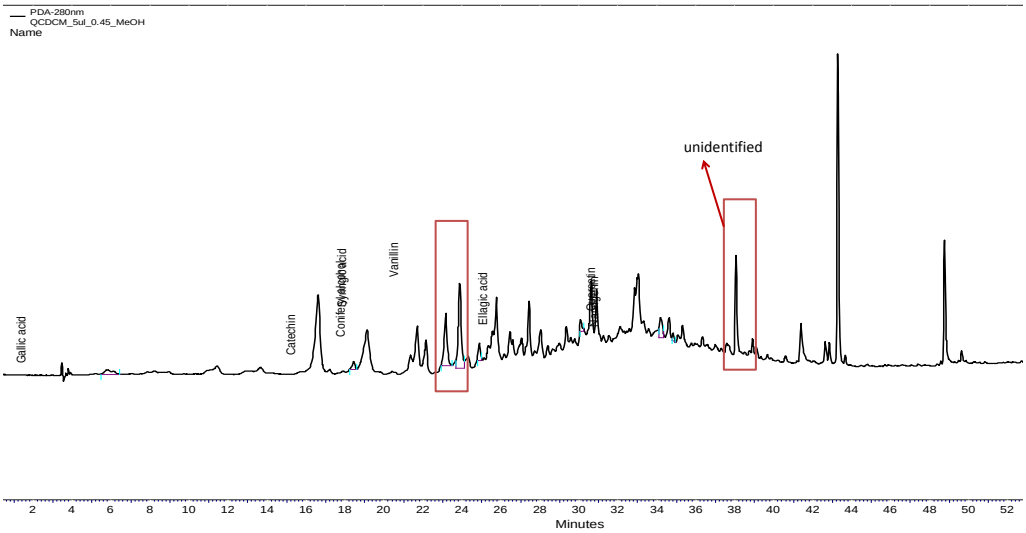
The proposed work to develop in this STSM with the duration of 1 month was very ambitious. Defining a specific HPLC method, choosing the right elution gradient, the right mobile phases and column it is not something particularly easy to do. But thanks to the expertise of the members of the host institution and our knowledge of the chemical composition of the prepared extracts, this STSM was really interesting not only for the preliminary results achieved or for the knowledge that was transmitted between both groups, but mainly because it was the beginning of a collaboration between institutions that, for now, will bring a more detailed knowledge of the chemical composition of the different bark polar extracts studied, allowing the study of this residues (barks are among the main residues from wood and cork industries) to increase their potential added-value.

The main conclusions of this STSM results are:

Pseudotsuga menziesii	
Bark	-bark extract have to major compounds: eriodictyol and an unidentified compound at Rt=27 min; -both eriodictyol and an unidentified compound were identified in cyclohexane, dichloromethane and ethyl acetate fractions; -the dichloromethane fraction is richer than the other fractions analyzed, and besides the unidentified compound and eriodictyol, naringenin is also very abundant;

	-were also identified, as minor compounds, syringic acid, vanillin, quercetin, gallic acid, catechin, epicatechin, coniferyl alcohol and ellagic acid.  **Figure 8 - Chromatogram of dichloromethane fraction of P. menziesii bark extract**
Cork	-cyclohexane fraction was very poor in compounds, and only syringic acid, vanillin, eriodictyol, quercetin and naringenin were identified as minor compounds; -the major compound in the cyclohexane fraction is the unidentified compound that elutes around 27 min; -the dichloromethane fraction is rich in semi-polar compounds: the major compounds are still the unidentified around 27 min, eriodictyol, naringenin, quercetin, syringic acid, and two more that we could not identify; -the ethyl acetate fraction is mainly constituted by the unidentified compound at 27 min, but it has also small amounts of eriodictyol, naringenin, quercetin, ellagic acid and vanillin.
Phloem	-in the cyclohexane fraction we were able to identify as minor compounds vanillin, catechin, naringenin and eriodictyol, but the major compound was not identified (Rt = 27 min); -the dichloromethane extract is again the most richer extract, being eridictyol the major compound, next to the unidentified (eluted at 27 min) and naringenin; - as minor compounds were also identified in the dichloromethane extract gallic acid, catechin, syringic acid, vanillin, coniferyl alcohol, and quercetin; -ethyl acetate fraction is poor in compounds: we identified as minor compounds only catechin, epicatechin, eriodictyol and quercetin – the major

	compounds are unidentified (eluted at 27 min, 37 min and 39 min).  **Figure 9 - Chromatogram of dichloromethane fraction of P. menziesii phloem extract**
Betula pendula	
Bark	-bark ethanol extract from *B. pendula* is very rich in polar compounds; -as minor compounds we identified catechin, syringic acid, vanillin, ellagic acid, quercetin and naringenin; -Unfortunately, the major compounds eluted at around 24 min, 29 min, 31 min and 39 min.
Quercus cerris	
Bark	-the cyclohexane and ethyl acetate fractions are not rich in polar compounds being only identified some minor compounds as syringic acid, vanillin and ellagic acid (gallic acid was the ajor compound in ethyl acetate fraction); -dichloromethane extract is very rich in semi-polar compounds: we were able to identify gallic acid, catechin, syringic acid, vanillin, coniferyl alcohol, ellagic acid, quercetin and naringenin; - syringic acid and vanillin were identified as the major compounds of this extract, but also a compound eluted at around 44 min.
Quercus suber	
Bark	-the cyclohexane fraction was only constituted by a single compound: vanilin (in small amount) – it was a very poor fraction; -in the dichloromethane fraction we were able to identify catechin, syringic acid, vanillin, coniferyl alcohol and ellagic acid; -the major compound of the dichloromethane fraction eluted at 44 min but we were not able to identify it; -ethyl acetate extact was also very poor, being only identified gallic acid,

	syringic acid, vanillin and querectin in very small amounts and a major compound at 31min that we still were not able to identify.  **Figure 10 - Chromatogram of dichloromethane fraction of Q. cerris bark extract**
Quercus variabilis	
Bark	-all the fractions extracted and collected from the bark of this specie were very poor in polar compounds able to elute: we identified catechin, epicatechin, vanillin, syringic acid, coniferyl alcohol, ellagic acid and quercetin, but all in very small quantities.

Final remarks:

Some of the extracts or fractions from the extracts studied e.g. *Q. variabilis* seem to be very poor in polar compounds. However this conclusion is not entirely true because we tested the presence of phenols, flavonoids and tannins and they exist in all extracts in high concentrations. This affirmation only means that we were not able to see peaks in the chromatograms. It could mean that, if these phenolics and/or flavonoids are linked to glycosides, they will not elute from the column easily. This affirmation is also supported not only by literature, but also by the experience with the TLC analysis we did to the fractions of the different extracts samples. The samples did not eluted from the application point meaning that they are too polar and that they have high affinity to the stationary phase, or the molecules could not pass through the column filling porous, or even because the molar mass of the compounds are too high.

One of the possible solutions for this is trying the separation by GPC that will be performed by the host institution and the other possibility might be a separation by

column chromatography accomplished by me in my institution, before a new analysis in HPLC-UV and HPLC-MS.

4. Future collaboration with host institution

Both institutions (Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Portugal and the host Department of Wood Technology, Biotechnical Faculty, University of Ljubljana, Slovenia) will still collaborating in the development of the work initiated in this STSM.

4.1. Quantification

In this moment, we are validating the method for a correct quantification of the compounds identified. Both institutions are still analyzing the same samples by HPLC-MS in order to identify some of the major compounds found, for example, in *P. menziesii* bark, cork and phloem portions, in *B. pendula*, in *Q. suber* ethyl acetate extract, and some minor compounds in *Q. cerris* and *Q. variabilis* dichloromethane bark extracts. Mass spectra will undoubtedly characterize the peaks we still need to identify, and then we can acquire the corresponding standards and have the full identified chromatograms.

4.2. GPC analysis

Separations based on size like gel-permeation chromatography (GPC) are based in the discovery of Porath and Flodin, by passing (filtering) them through a controlled-porosity, hydrophilic dextran polymer. This process was termed gel filtration. Later, an analogous scheme was used to separate synthetic oligomers and polymers using organic-polymer packings with specific pore-size ranges. This process was called gel-permeation chromatography (GPC). Similar separations done using controlled-porosity silica packings were called size-exclusion chromatography (SEC). Introduced in 1963, the first commercial HPLC instruments were designed for GPC applications.[32] All of these techniques are typically done on stationary phases that have been synthesized with a pore-size distribution over a range that permits the analytes of interest to enter, or to be excluded from, more or less of the pore volume of the packing. Smaller molecules penetrate more of the pores on their passage through the bed. Larger

molecules may only penetrate pores above a certain size so they spend less time in the bed. The biggest molecules may be totally excluded from pores and pass only between the particles, eluting very quickly in a small volume. Mobile phases are chosen for two reasons: first, they are good solvents for the analytes; and, second, they may prevent any interactions [based on polarity or charge] between the analytes and the stationary phase surface. In this way, the larger molecules elute first, while the smaller molecules travel slower [because they move into and out of more of the pores] and elute later, in decreasing order of their size in solution. Hence the simple rule: big ones come out first.[40,41]

So, in the case of our samples from *B. pendula, p.menziesii* and specially in *Q. suber, Q. cerris* and *Q. variabilis*, we were not be able to see as many peaks as we were expecting, according to the content of phenolics, flavonoids and tannins determined for each samples. We think that is probably due to the fact that normally flavonoids occur naturally as glycosides [42,43]. This problem was also proved when we tried to analyse the samples by thin layer chromatography (TLC) and they did not eluted from the application point using several elution systems as dichloromethane:ethanol 3:7, dichloromethane:ethyl acetate 2:8, ethanol:ethyl acetate 4:6 or even dichloromethane:ethyl acetate 1:9 with a few drops of pyridine. We concluded that the compounds present in the polar extracts have high affinity for stationary phase (silica on the column and also on plates) so they must be were big molecules, with high molecular weight and, as mentioned above, phenols like flavonoids must occur as glycosides.

This problem will be solved during our collaboration with the host institution that will use GPC (gel-permeation chromatography) to separate the compounds by size and analyze the mass distribution in each extract and corresponding fractions.

5. Foreseen publications/articles to result from the STSM

According to the preliminary results obtained with this STSM (1 month) we are expecting to describe the most interesting results in three papers:

- one with the full qualitative and quantitative characterization of the polar extracts of *B. pendula*, but of course we will need to analyze the sample also in HPLC-MS to identify the other compounds that we could not identify without standards and also to have a more consistent analytical writing;

- one with a comparative study of the composition of ethanol bark extracts from the different *Quercus* species in study and that will include not only the work done during the STSM but also the GPC studies that are undergoing in the host institutuion;
- another with the full qualitative and quantitative characterization of the polar extracts of *P. menziesii*, also after a more detailed characterization, with HPLC-MS.

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