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Gram-Scale Purification of Dihydrorobinetin from *Robinia pseudoacacia* L. Wood by Centrifugal Partition Chromatography

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Abstract: *Robinia pseudoacacia* L. is a tree widely dispersed in France that is characterized by good growth rates and important biomass production, which produces wood with very high natural durability used for outdoor fence posts, timber, and barrels to age vinegars and wines. Its mature heartwood presents high resistance against wood fungi decay and contains two main flavonoid extractives, dihydrorobinetin—the most abundant—and robinetin that present interesting biological activities. The aim of the present study was to optimize a procedure allowing an important recovery of purified dihydrorobinetin from *R. pseudoacacia* wood, representing an interesting sustainable, local, highly available, and, consequently, economical source of bioactive components. The extraction of dihydrorobinetin was first optimized by evaluating the influence of various extraction parameters such as temperature, extraction time, solvent nature, and wood/solvent mass ratio to obtain an efficient, safe, and low cost extraction. Then, dihydrorobinetin was purified over 95% using centrifugal partition chromatography (CPC). CPC purification was first developed on a small volume column with low amounts of injected extract, then scaled-up on a 200 mL column with higher sample loading capacity in order to purify more than 1.3 g of dihydrorobinetin in one run.

Keywords: centrifugal partition chromatography; mass spectrometry; flavonoids; *Robinia pseudoacacia* wood extracts; dihydrorobinetin

1. Introduction

Black locust (*Robinia pseudoacacia* L.) belongs to the Fabaceae family. Originating from southeastern North America this leguminous tree species was introduced in France in 1601 by Jean Robin and has since then extensively spread within the temperate regions of North America, Europe, Southern Africa, and Asia. With more than 3.2 million hectares, it is the third species used for wood production after poplars and eucalyptus [1]. Its rapid growth rate and the production of a large quantity of prolifically dispersed propagules allow black locust to successfully establish itself over a wide range of environmental conditions. Once established, *R. pseudoacacia* increases soil nitrogen stores [2]. However, it has been included on the European list of the most dangerous invasive species due to its significant regeneration abilities and its fast growth rate [3].

From vegetative and reproductive organs of the plant (flowers, leaves, seeds) flavonoid derivatives can be extracted [4,5]. These flavonoids have shown interesting biological properties such as inhibitory activities on tyrosinase, α-amylase and α-glucosidase [6], antioxidant and antimicrobial potentials [7,8], antibacterial effects against oral pathogens [9], bioactivities in the brine shrimp lethality test (BST), cytotoxicities against a panel of six solid human tumor cell lines [10], and protective effects against the cucumber powdery mildew fungus [11].

Interestingly, *R. pseudoacacia* wood has long been used to age vinegars (e.g., Modena balsamic vinegar) and, more recently, wine [12]. The air transfer efficiency through the pores of this wood favors efficient acetification rates and affects the phenolic composition and sensory quality of vinegar. Indeed, it has been observed that vinegars and wines aged in *R. pseudoacacia* wood barrels contained a characteristic compound, dihydrorobinetin, whose concentration increased during the aging process and could be used for authenticity purposes [12–14].

Characterized by good growth rates and important biomass production, *R. pseudoacacia* produces wood of very high natural durability used for outdoor fence posts, but also for timber (e.g., beam, railroad sleepers) [15,16]. Interestingly, the major proportion of its wood corresponds to heartwood (last differentiation step of wood cells) that retains most of its properties due to the accumulation of specific extractives as phenolic substances that increase the natural color and durability of wood [17]. Difference was observed between the mature and juvenile heartwood that presents lower resistance against wood fungi decay. Two flavonoids dihydrorobinetin (3,3′,4′,5′,7-pentahydroxyflavanone, DHRob) and robinetin (3,3′,4′,5′,7-pentahydroxyflavone, Rob) are the major phenolic compounds that have been extracted from *R. pseudoacacia* heartwood [18–20]. They are present in higher contents in mature heartwood compared to juvenile wood, and have thus been related to the higher decay resistance observed in mature heartwood [16,21,22].

In this context, it seemed interesting to use *R. pseudoacacia* wood as a promising sustainable local, highly available, and economical source of bioactive components. In this article, we particularly focused on dihydrorobinetin (DHRob) which is the main constituent of this resistant heartwood and that could be eventually valorized as a natural phytochemical protective for many applications in various activity sectors (pharmaceutics, cosmetics, phytosanitary, etc.). Thus, the aim of the present study was to develop and optimize, for the first time, a simple and efficient procedure allowing the important recovery of purified DHRob from *R. pseudoacacia* wood. An easily transferable to pilot scale extraction process was first optimized by evaluating the influence of various extraction parameters like temperature, extraction time, solvent nature, and wood/solvent mass ratio. Then, centrifugal partition chromatography (CPC), a liquid–liquid partition method [23,24], was used to purify dihydrorobinetin at gram-scale.

Until now, most of the chromatographic methods used to perform gram-scale purifications of natural compounds were based on solid support (i.e., medium performance liquid chromatography [25], flash chromatography [26], preparative liquid chromatography on macroporous resin [27] or on reversed phase column [28], and supercritical fluid chromatography [29]). Interestingly, CPC separations rely on the use of a liquid stationary phase that presents numerous advantages such as (i) no irreversible adsorption of samples; (ii) no compound degradation; (iii) high column loading capacity; (iv) high sample recovery; (v) lower cost; and (vi) shorter separation time [30,31]. Hence, this technique is particularly well adapted to the purification of various natural compounds from mg to g scale with standard laboratory equipment [32–34].

2. Materials and Methods

2.1. Plant Material

One *Robinia pseudoacacia* L. tree (of about 25 years) from on the nursery of INRA Orleans Center was felled in winter, manually debarked, grinded with a wood pellet mill (Noremat B250, Ludrus, France), and stored at room temperature in wood crates in a ventilated warehouse until use. These pellets were then grinded (SM 2000, Retsch, Haan, Germany) and sieved with AS 200 (Retsch,

Haan, Germany) to obtain homogenous wood particles averaging 0.6 mm. Extremely fine wood powder was discarded (filtered out) to avoid further interferences with the extraction process. The size selected wood particles were freeze-dried (Christ alpha 1–4 lyophilisator) (Grosseron, Coueron, France) just before extraction.

2.2. Chemicals

All solvents: ethanol (EtOH), ethyl acetate (EtOAc), methanol (MeOH), heptane (hept) and acetonitrile (ACN) were of analytical grade and purchased from VWR (Fontenay-sous-Bois, France). Water was purified (resistance < 18 MΩ) by an Elgastat UHQ II system (Elga, Antony, France).

Standard molecules robinetin (Rob, purity HPLC $\geq$ 99%) and dihydrorobinetin (DHRob, purity HPLC $\geq$ 95%) were obtained from Extrasynthese (Genay, France) and used as internal controls. Structures of Rob and DHRob are presented on Figure 1.

2.3. Extraction Method

The solid–liquid extraction method was adapted from methods already described [13,20] to improve DHRob extraction. Extraction was carried out in a batch reactor of 1.5 liter. The reactor was connected to a thermocryostat allowing the temperature control in the studied domain. It was equipped with a stirring system to enable rotation speed of up to 2000 rpm, permitting the homogenization of the heterogeneous solution. Different wood extraction conditions were tested successively: (i) EtOH/H_2O ratios as solvent mixture; (ii) extraction kinetic from 1 to 24 h of maceration under 550 rpm agitation; (iii) temperature from 10 to 40 °C, and wood/solvent mass ratio ($R_{w/s}$) from 1% to 13%. After removal of the wood by filtration with a nylon mesh, the extract was evaporated under vacuum with a rotary evaporator (Büchi, Flawil, Switzerland) at temperature below 40 °C to obtain a dry crude extract. During the evaporation step, the ethanol evaporated first leading to the precipitation of less water-soluble compounds. Separation of the two phases was realized by centrifugation at 10,000 rpm for 10 min. The concentrated brown liquid aqueous phase (named S) contained the most polar compounds and the yellow solid phase (named D) contained the less polar compounds. After separation both phases (S and D) were dried under vacuum.

2.4. HPLC Analysis

All extract analyses were performed using a LaChrom HPLC-Diode Array Detector instrument controlled by EZChrom Elite workstation software (VWR, Fontenay-sous-Bois, France). The DAD was set from 200 to 600 nm to record absorbance spectra (Figure 1). Chromatograms were visualized at 280 ($\lambda_{maxDHRob}$), 310 (equivalent absorbance of DHRob and Rob) and 366 nm (λ_{maxRob}).

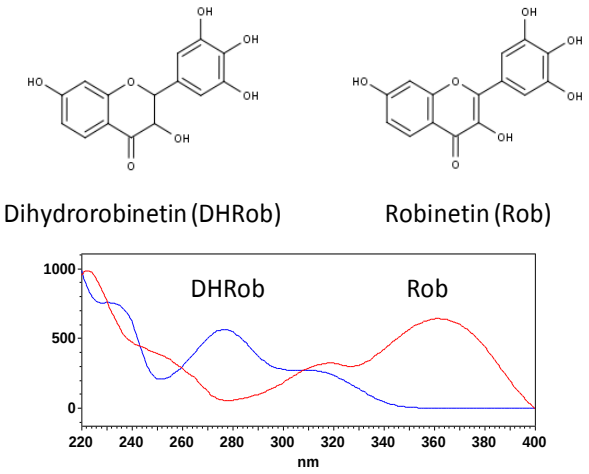

Figure 1. Dihydrorobinetin (DHRob) and robinetin (Rob) structural formulae and absorbance spectra: blue line (DHRob), red line (Rob).

The acquisition system used was the Ezchrom software, 3-2-1 version. Separation of flavonoids was performed using reversed-phase liquid chromatography [20]. The column used was an Altima C18 (Grace, Epernon, France), 150 mm × 4.6 mm, with a particle size of 5 µm. The mobile phase at a flow of 1 mL·min^{-1} was made up of 0.1% formic acid in water (phase A), and 0.1% formic acid in methanol/acetonitrile (50/50) (phase B). A gradient of solvents was applied as follow: 0–15 min: 5%–25% phase B, 15–24 min: 25%–100% phase B, and finally 24–25 min 5% phase B, maintained during 10 min before each new injection. The column was introduced in an oven Jetstream and heated at 25 °C. The injection volume was 20 µL. Quantitative analyses of DHRob and Rob were performed by injecting standard solutions at different concentrations from 10 mg·L^{-1} to 500 mg·L^{-1}. Two calibration curves obtained with 5 standard points for DHRob at 280 nm ($Y = 22.084x + 91.996$, $R^2 = 0.9997$) and for Rob at 366 nm ($Y = 46.165x - 254.45$, $R^2 = 0.9992$) were used to estimate the respective amounts of DHRob and Rob in the extracts. For each analysis, samples were diluted into the HPLC mobile phase used at the beginning of the elution gradient before injection to obtain DHRob and Rob peak areas within the range defined by the calibration curves.

2.5. HPLC-MS Analyses

The HPLC-MS system consisted of an UltiMate 3000 RSLC apparatus coupled with a TSQ Endura triple quadrupole mass spectrometer equipped with a heated electrospray ionization (H-ESI) interface (Thermo Scientific Inc., San Jose, CA, USA). Molecules were separated on a Luna C8 column (150 mm × 4.6 mm) with a particle size of 3 µm (Phenomenex, Le Pecq, France). The column temperature was maintained at 30 °C. Mobile phase was composed of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Elution gradient was recorded 0–10 min: 10% solvent B, 10–25 min: 10%–20% solvent B, 25–35 min: 20%–100% solvent B at 0.8 mL·min^{-1}, and 10 min of equilibration time between two injections. Injection volume was 2 µL.

The MS parameters applied were: Electrospray Ionization (ESI) ionization source, the analytes were monitored under negative mode, electrospray voltage of −2200 V, vaporizer temperature of 317 °C, ion transfer temperature of 333 °C, sheath gas of 20 Arb, auxiliary gas of 5 Arb, sweep gas of 3 Arb. Mass spectra were recorded in the range of 100–1000 *m/z*.

2.6. Centrifugal Partition Chromatography Purification

The DHRob purification method was developed with a semi-preparative FCPC® from Rousselet Robatel Kromaton (Annonay, France). The mobile phase was pumped by an Agilent Technologies 1100 Series binary pump (Palto Alto, CA, USA). The sample was introduced in the column using a 6-port high pressure Rheodyne injection valve (AIT, Houilles, France) equipped with a 10 mL loop. The CPC instrument was coupled with a UV detector (Spectroflow 783 model) from Kontron Instruments (Montigny Le Bretonneux, France) set at 310 nm and with an Evaporative Light Scattering Detector (ELSD) (SEDEX 55 from SEDERE). CPC effluents were not all directed through detectors but split with a variable flow splitter from Rheodyne (Rohnert Park, CA, USA). This device transfers a small volume of CPC effluents into a separate and independent auxiliary stream directed to UV and ELSD detectors [35]. The auxiliary stream was supplied by a one-way LC-10 AS pump (Shimadzu, Kyoto, Japan) delivering ethanol at a flow rate of 0.3 mL·min^{-1}. The parts not switched through the detection device were collected into 10 mL collecting tubes that were individually subjected to HPLC analysis in order to pool fractions with similar fingerprints.

DHRob purification at gram scale was performed on a 200 mL hydrostatic column with an ethyl acetate/methanol/water (1:0.05:1 *v*/*v*/*v*) biphasic system. Rotation speed was 1400 rpm and mobile phase flow rate was 3 mL·min^{-1}. In these conditions, 70% of the stationary phase retention was observed and 5 g of the dried S phase diluted in 8 mL of the biphasic system could be injected. Elution was managed in descending mode (the organic phase was used as stationary phase while the aqueous phase was used as the mobile one) then the whole content of the CPC column was extruded by pumping the organic phase in descending mode at 5 mL·min^{-1} and 500 rpm [36].

3. Results and Discussion

3.1. Extraction Optimisation

To determine an efficient extraction of DHRob from *R. pseudoacacia* L. wood, different extraction parameters were optimized (solvent nature, $R_{w/s}$ mass of wood/solvent ratio, extraction time, and temperature). Using the same amount of starting material, extraction efficiency was compared after HPLC analysis through the estimation of DHRob concentration in each extract using calibration curve obtained with DHRob standard molecule. The different extraction parameters tested and the corresponding DHRob concentrations are reported in Table 1. Preliminary experiments (adapted from [20]) comparing H_2O, EtOH 80%, Acetone 80%, and MeOH 80%, showed an efficient DHRob extraction with EtOH/H_2O 80/20 with a concentration of 870 mg·L^{-1}. Taking into account extraction, toxicity, safety, and cost issues related to the other organic solvent, an ethanol-based solvent was selected to study the others parameters to be optimized (Table 1).

Table 1. Extraction parameters optimization according to DHRob concentrations (in bold selected parameters for the next optimization step).

Parameter	Value	DHRob Concentration mg·L^{-1}
Preliminary solvent screening	H_2O	240
	MeOH/H_2O 80/20	820
	EtOH/H_2O 80/20	**870**
	Acetone/H_2O 80/20	780
Optimization in EtOH/H_2O 80/20		
Temperature	15–40 °C	740 ± 29
$R_{w/s}$	1%	120
	5%	770
	9%	1380
	13%	**1782**
Time	1 h	1050
	2 h	1500
	4 h	**2000**
	6 h	1950
	24 h	2023
Optimized extraction		
	EtOH/H_2O 50/50	**2500**
	Sphase	38%
	Dphase	5.5%

The results show that different temperatures of extraction between 15 and 40 °C have no influence on DHRob extraction yields, as the average value of DHRob concentration was equal to 740 mg·L^{-1} with a standard deviation of 29 mg·L^{-1}. Higher temperatures were not tested in order to avoid any risk of compound degradation and also to limit global energy consumption. Thus, room temperature of about 25 °C was selected and further used. As for the wood mass/solvent ratio, the extraction of DHRob appeared to increase proportionally to the vegetal mass introduced in the reactor. The ratio $R_{w/s}$ varied from 1% to 13%. Indeed, for higher ratios the sample agitation process was hindered due to the high amount of raw material in the reactor. In the end, a $R_{w/s}$ of 13% was selected as the optimum mass/solvent ratio. The extraction kinetic was then followed by analyzing DHRob concentration in the extract every hour for 6 h and, lastly after 24 h. An equilibrium was achieved after 4 h. Interestingly, no compound degradation could be recorded even after 24 h. The value of DHRob concentration obtained was equal to 2023 mg·L^{-1}.

Solvent composition was then refined to improve specifically DHRob extraction by testing different EtOH/H_2O volume proportions from 0% to 100%. To determine the most effective solvent ratio, extracts were analyzed by HPLC. The recorded chromatograms for EtOH/H_2O 80/20, EtOH/H_2O 70/30 and EtOH/H_2O 50/50 are presented in Figure 2. The relatively higher DHRob peak

area suggested a better efficiency of the 50/50 EtOH/H_2O extraction solvent that favored the DHRob extraction. Moreover, the use of a lower EtOH content improves extraction safety and should decrease the extraction cost once transferred at an industrial level.

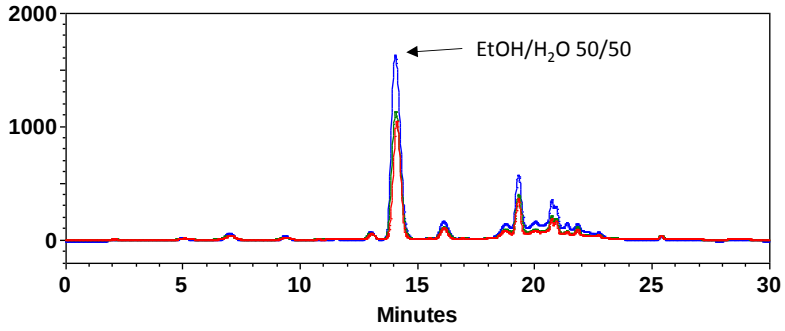

Figure 2. Chromatograms of crude extracts UV detection at 280 nm for three extraction solvent compositions EtOH/H_2O 80/20 (red), EtOH/H_2O 70/30 (green), and EtOH/H_2O 50/50 (blue).

In conclusion, the best conditions for DHRob extraction were defined as following: solvent 50/50 EtOH/H_2O, wood powder/solvent mass ratio $R_{w/s}$ of 13%, 4 h at room temperature 25 °C.

3.2. HPLC Analysis of the Optimized Extract

Dihydrorobinetin (DHRob) and robinetin (Rob) have been described as the main characteristic compounds found in *R. pseudoacacia* wood extracts [20,21]. Standard molecules (DHRob and Rob) injected in the HPLC device were eluted at 14.1 min and 20.8 min respectively. Figure 3 shows typical crude extract HPLC-DAD chromatograms recorded at 3 wavelengths (280, 310, and 366 nm, Figure 1). HPLC-DAD analysis allows the detection of phenolic compounds present in the extracts and reveals the presence of two main compound families. The first one corresponds to the most polar compounds. It mainly absorbs at 280 nm and presents retention time and UV spectrum close to the DHRob standard. The second molecular group, less polar, is more similar to the Rob reference characterized by a longer retention time and UV spectrum presenting a maximum absorption of about 370 nm.

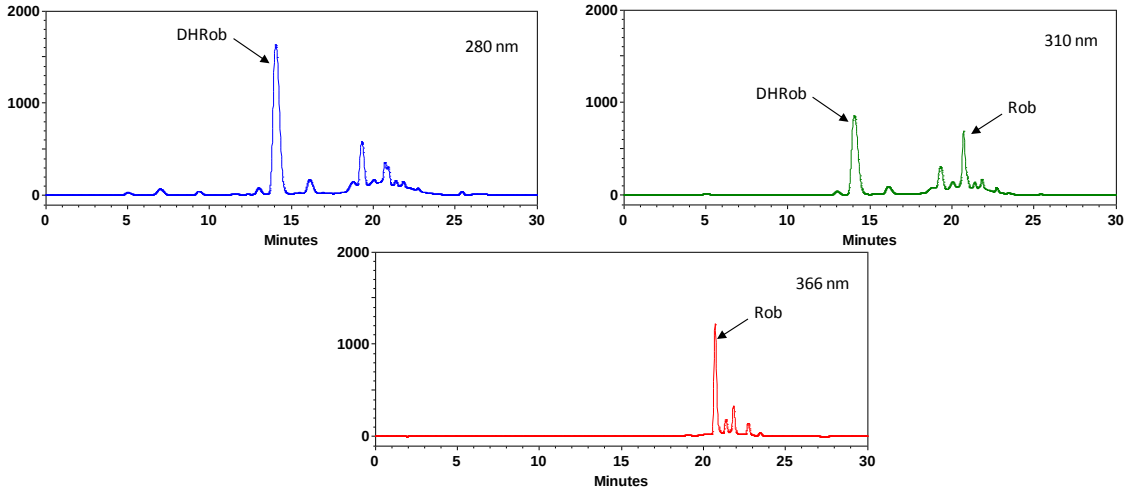

Figure 3. Chromatogram of optimized crude extract UV detection at 280, 310, and 366 nm.

Calibration curves respectively calculated for DHRob and Rob were used to estimate a content of 2500 mg·L^{-1} of DHRob and 600 mg·L^{-1} of Rob in the crude extract just after raw material filtration. During the initial phase of concentration (EtOH evaporation), precipitation of less polar compounds

(principally Rob) occurred, leading to the formation of two phases: a liquid aqueous brown phase (S phase) and a yellow solid phase (D phase). The amounts of DHRob and Rob were estimated in both of these phases in order to follow the partition of the two compounds. The S phase contained 38% of concentrated DHRob and 2% of Rob while the D phase contained only 5.5% of DHRob and in an opposite manner 34% of Rob (Table 1).

3.3. DHRob Purification by Centrifugal Partition Chromatography

In an attempt to develop a gram scale purification method of DHRob by the mean of centrifugal partition chromatography (CPC) several set-ups were carried out. The method was developed first on small 50 mL column in order to perform a faster method development and to limit extract and solvent consumption. Then, purification was scaled-up with a 200 mL column, allowing higher amounts of sample to be loaded. The different results of DHRob CPC purification are summarized in Table 2.

At first, small amounts of crude extract were used to determine the partition coefficient of the two main compounds DHRob and Rob. Two milligrams of crude extract were dissolved in a biphasic system (4 mL) in a test tube and both phases were analyzed by HPLC-DAD. According to previous solvent systems used for flavonoid purification [37–39], the various tested biphasic systems are composed of solvents such as heptane, methanol, ethanol, ethyl acetate, and water in different proportions. Among all these systems, the best partition was obtained with the Arizona G [40] system: heptane/ethyl acetate/methanol/water (1:4:1:4, *v*/*v*/*v*/) with K_{DHRob} = 1.4 and K_{Rob} = 3.8. DHRob purification was then evaluated on a small volume column of 50 mL from only 35 mg of crude extract. DHRob was conveniently eluted in descending mode between 25 and 40 min at 2 mL·min^{-1} with a rotation speed at 2000 rpm. Fraction analysis showed a satisfying DHRob purification with these conditions with a DHRob recovery of 11.3 mg. So, purification was restarted, increasing the injected amount. Five hundred milligrams of crude extract were gradually dissolved in 4 mL of the biphasic system. The aqueous phase appeared limpid with a good solubility of polar compounds. However, the organic phase remained very opaque, forbidding sample injection. Thus, the solubility of the crude extract in the Arizona G system was too low to increase sample loading and improve the amount of recovery of purified DHRob. As heptane could limit sample solubility, different ethyl acetate/water/methanol systems were re-evaluated. In the end, an ethyl acetate/methanol/water (1:0.05:1 *v*/*v*/*v*) system was selected due to the fact that K_{DHRob} = 3.2 and Rob was quantitatively retained in the organic phase. With this system, DHRob would be more retained in the stationary phase in order to improve its purification in spite of the higher injected amount. Rob was strongly retained in the stationary phase but would be extruded at the end of the separation to reduce the total analysis time.

To improve sample solubility, loading capacity, and DHRob purification yields, the DHRob-enriched S phase was used as starting sample. Four hundred and ninety-five milligrams of the S phase could properly be dissolved in 4 mL of biphasic system and injected in the CPC device. Using the previously determined elution conditions, DHRob was observed between 50 and 80 min. After fraction evaporation, a total of 170 mg of purified DHRob (93% purity) was recovered.

As a final step, the DHR purification process was scaled-up by using a 200 mL CPC column and the same biphasic system. Operating conditions were adapted to the 200 mL column: rotation speed was 1400 rpm and mobile phase flow rate was 3 mL·min^{-1} in descending mode. Seventy percent of stationary phase retention was obtained. Five grams of the S phase were successfully dissolved in 8 mL of the biphasic system and injected. Taking into account the column volume ratio between both columns used (50/200) and the average of loading sample (7.3 mg/column·mL) commonly used in CPC [32,34] this represents a high sample loading, limited by the solubility of the sample in the biphasic system. The CPC chromatogram obtained is presented on Figure 4.

Table 2. Development steps for gram-scale dihydrorobinetin (DHRob) purification.

CPC Column	Biphasic System	Partition Coefficient	Sample Loading	DHRob Recovery
50 mL	Hept/EtOAc/MeOH/H_2O 1:4:1:4	K_{DHRob} = 1.4 K_{Rob} = 3.8	35 mg crude extract	11.3 mg
50 mL	Hept/EtOAc.MeOH/H_2O 1:4:1:4		500 mg crude extract Low solubility	-
50 mL	EtOAc/MeOH/H_2O 1:0.05:1	K_{DHRob} = 3.2 K_{Rob} = ∞	495 mg S phase	170 mg purity 93%
200 mL	EtOAc/MeOH/H_2O 1:0.05:1		5 g S phase	1.308 g purity 95% 0.675 g purity 85%

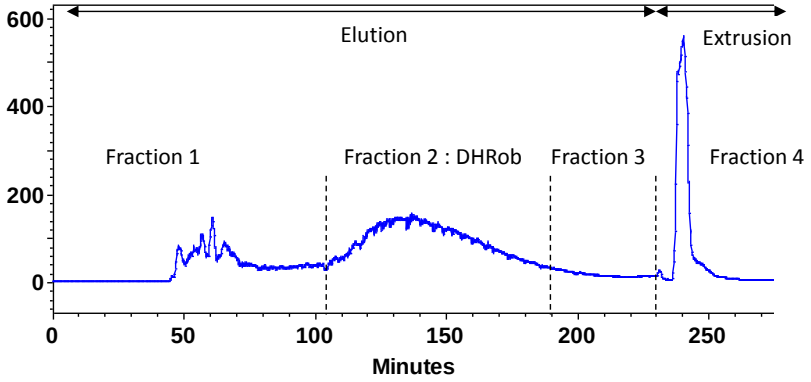

Figure 4. Centrifugal partition chromatography-Evaporative Light Scattering Detector (CPC-ELSD) chromatogram CPC column 200 mL, rotation speed 1400 rpm, biphasic system ethyl acetate/methanol/water (1:0.05:1 $v/v/v$) at 3 mL·min^{-1} in descending mode.

DHRob was eluted between 105 and 180 min. Other collected tubes were gathered in three different fractions. The first one (fraction 1) contained the most polar compounds eluted before DHRob, fraction 3 contained compounds eluted in the mobile phase after DHRob, and the last one (fraction 4) is composed of compounds extruded in the stationary phase (Figure 4).

According to the HPLC fraction analysis, two DHRob fractions shown in Figure 5 could be obtained. The first one composed of the tubes collected between 105 and 150 min contained 1.308 g of DHRob purified over 95% representing 26% of the injected sample amount. The second one (tubes collected from 150 to 180 min) contained 0.675 g of DHRob purified at 85% representing 13% of the injected sample. In the end, all the DHRob contained in the initial sample appeared to be totally recovered through the established CPC process as the total amount recovered represented 39% of the injected sample when HPLC quantification of DHRob in the injected S phase indicated a proportion of 38%.

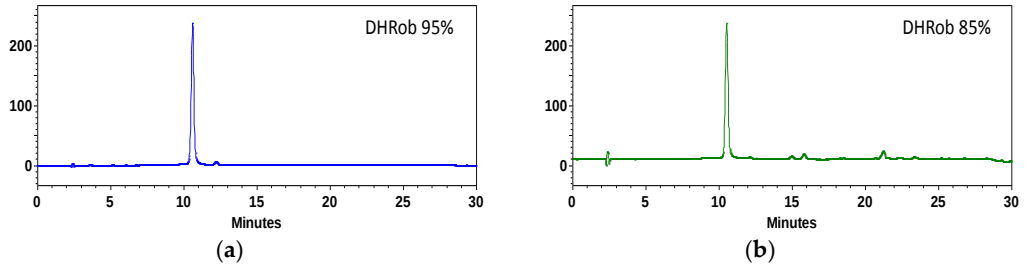

Figure 5. HPLC chromatograms of the two purified DHRob fractions at 95% (**a**) and 85% (**b**). UV detection at 280 nm.

3.4. HPLC-MS Analysis of CPC Fractions

The four CPC fractions were analyzed using HPLC-MS to validate the presence in our extract of other known compounds frequently found in *R. pseudoacacia* extracts as well as to follow their partitioning in the different fractions. Table 3 summarizes the detection of compounds that have already been described according to their absorbance and mass spectra [13,19,41,42].

The first fraction contained mainly molecules with absorbance maxima between 280 and 320 nm and with low molecular mass eluted at the beginning of the HPLC-MS chromatogram. Fragment ions at *m/z* 167, 301 and 285 were the base peak of the mass spectra and neutral loss of H_2O (−18u) were frequently observed. These compounds could be assigned as leucorobinetinidin isomers and hydroxydihydroflavonol derivatives. Other compounds with higher molecular mass were eluted in the same CPC fraction. UV spectra of these compounds showed also absorbance maxima at 280–310 nm. Mass spectra base peaks at *m/z* 589, 603, 605 and 607 seemed to be the deprotonated molecule ion $[M-H]^-$. Fragmentation of these $[M-H]^-$ allowed the detection of fragment ions with low intensity at *m/z* 421, 301, 287. Even if the structures of these molecules were not fully determined they could be correspond to dimeric prorobinetinidin condensed tannins. These tannins eluted in the first CPC fraction, appeared on the HPLC-MS chromatogram between 21 and 25 min after the DHRob showing the difference of selectivity between these two chromatographic partition systems.

The second CPC fraction contained the purified DHRob. Purity of DHRob was estimated at 95% and the compound detected with DHRob appeared be to a DHRob isomer presenting the same absorbance and mass spectra with λ_{max} 280–310 nm and $[M-H]^-$ 303 *m/z* and $[M-H_2O-H]^-$ 285 *m/z*.

Table 3. HPLC-MS analyses of CPC fractions. HPLC Retention Time (RT); Absorbance spectra maxima (UV); Mass spectra main ions (MS).

Fraction	RT (min)	UV (nm)	MS (*m/z*)	Compound
1	3.4	281–320	**181**	Hydroxycinamic acid derivative
	4.3	280	303, 285, **167**, 137	Leucorobinetinidin isomer
	5.3	280	303, 285, **167**, 137	Leucorobinetinidin isomer
	9.4	280	319, **301**	Pentahydroxydihydroflavonol
	10.6	280–320	303, **285**	Tetrahydroxydihydroflavonol
	21.6	288	**589**, 449, 301	Dimeric prorobinetinidin
	22.4	280–310	**605**, 421	
	23.2	280–310	**589**, 419	Dimeric prorobinetinidin
	23.9	280–310	**603**, 585, 567, 457	
	24.1	290	**607**, 467, 301	
2	14.8	280–310	303, **285**	Dihydrorobinetin
	17.1	280–310	303, **285**	Tetrahydroxydihydroflavonol
3	27.9		**575**, 423, 287	
	28.8		**589**, 419	Dimeric prorobinetinidin
4	19.4	290	**319**	Pentahydroxydihydroflavonol
	21.9	290	**319**	Pentahydroxydihydroflavonol
	23.6	280–310	**317**, 299, 289, 284, 274	Trihydroxymethoxydihydroflavonol
	23.8	280–310	**287**, 269, 259, 243, 225	Fustin
	25.4	280–310	**287**	Robtin
	27.9	260–360	301	Robinetin
	29.1	260–396	**285**, 149	Tetrahydroxyaurone
	29.4	280–310	**271**	Butin
	30.0	260–384	**287**, 269, 151	Robtein
	30.3	280–310	**255**	Liquiritigenin
	30.9	260–380	**271**, 253, 135	Butein
	31.8	370	**255**	Isoliquiritigenin

CPC tubes collected between 175 and 230 min at the end of the DHRob elution constituted the fraction 3. HPLC-MS analysis of this fraction showed the presence of two other condensed tannins *m/z* 575 and 589 at low concentration.

Lastly, compounds eluted during the CPC extrusion phase were mainly flavanones, flavonols, and chalcones such as robtin, robinetin, butin, butein with absorbance spectrum with higher λ_{max} above 360 nm. Molecule deprotonated ions were the base peaks on mass spectra. Main fragment ions showed loss of H_2O (−18u). These less polar molecules than DHRob are eluted later in CPC and in HPLC-MS.

4. Conclusions

The extraction of dihydrorobinetin from *R. pseudoacacia* wood was first optimized leading to an efficient, safe and low cost process. Then, DHRob was successfully purified using centrifugal partition chromatography. Five grams of extract enriched in DHRob (through the precipitation and centrifugation of the less water-soluble compounds) were injected on a 200 mL CPC column. Thereafter, 1.3 g of DHRob (purity over 95%) and 0.675 g (purity over 85%) were recovered in one CPC run of 200 min. In the end, the quantity of DHRob that could be recovered represents 39% of the injected sample and 0.5% of the initial raw dried wood material. DHRob is now routinely purified with this optimized methodology to produce purified compound for collaborators that are comparing its biological activities to those of the crude extract.

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