HPLC ANALYSIS OF PHENOLIC COMPOUNDS IN NORWAY SPRUCE WITH PHOTODIODE ARRAY AND MASS SPECTROMETRIC DETECTION

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Abstract

This paper deals with phenolic compounds in Norway spruce needles and practical method for their determination. The importance of phenolic compounds in plants consists in their ability to absorb radiation just in the UV-B range and thus protect plants from its harmful effect. Identification of phenolic compounds according to their spectral properties was carried out by liquid chromatography. For quantification of phenolic compounds photometric signals at selected wavelengths measured on HPLC system Dionex with Chromeleon software were chosen. Three procedures were used in this study to isolate UV-light screening substances from Norway Spruce needles. In extracts from Norway spruce needles besides p-hydroxyacetophenone as major component and catechin, six phenolic acids were identified. However, in order to simplify chromatograms and eliminate interfering components, sample fractionation based on ion-exclusion or hydrophilic interactions is promising and will be a subject of further study.

Key words

Phenolic compounds, Norway spruce, needles method, determination, liquid chromatography, plants.

Introduction

Phenolic compounds are expected to play an important role in the protection of plants from UV radiation from the sun. This radiation is conventionally split into three main regions: UV-A, UV-B and UV-C. UV-A of the wavelength 320-400 nm reaches the surface of the earth relatively unattenuated and is probably nearly harmless. UV- B radiation in the range 280-315 nm is partially absorbed by earth's atmosphere. It extends to wavelengths where DNA absorbs. This radiation therefore is harmful as it can induce changes in genetic code of living organisms. The UV-C radiation of the wavelength 200-280 nm is extremely dangerous, but it is completely absorbed by atmospheric oxygen and ozone layer in the stratosphere [2].

Ozone filters out all the sun's ultraviolet light from 220 to 290 nm. However, its ability to absorb light in the 290 to 320 nm range is quite limited. The reduction in stratospheric ozone concentration allows more ultraviolet light of this range to penetrate to the earth's surface: it is predicted that 1 percent decrease in overhead ozone results in a 2 percent increase in ultraviolet B light intensity at ground level. This increase in ultraviolet B radiation is the reason for principal environmental concern of ozone depletion [1].

Directly observable adverse effects of UV-B radiation on plants are as follows: it causes decrease in photosynthetic activity, increases susceptibility of plants to decease, causes changes in plant structure and pigmentation and retards their growth [3].

The importance of phenolic compounds in plants consists in their ability to absorb radiation just in the UV-B range and thus protect plants from its harmful effect. The aim of this study was to identify and quantify such phenolic compounds in Norway spruce (*Picea abies)* needles and to develop practical method for their determination.

Experimental

Analysed compounds

Based on literature data, a series of phenolic compounds was selected which were reported to be present in Norway Spruce needles. This series included p-hydroxyacetophenone, benzoic acid, p-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, p-coumaric acid (Sigma), vanillic acid, catechin (Fluka) and ferulic acid (Aldrich). Their structural formulas are given in Fig. 1.

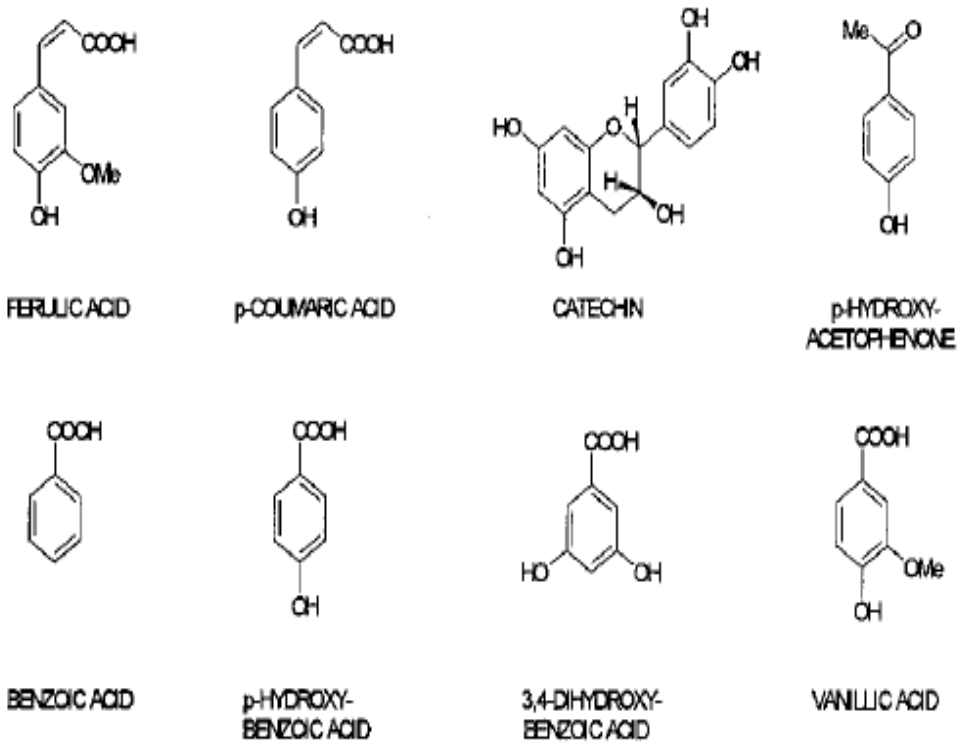

Fig. 1
Analysed compounds

Liquid chromatography

Identification of phenolic compounds according to their spectral properties was carried out by liquid chromatography (LC) with photometric diode array detector SpectroMonitor 5000 (LDC Analytical) working in the UV range (190-360 nm). For separation on a glass column (150 x 3 .2 mm) packed with Separon SGX C18, 7 µm (Tessek) a mixture of methanol - 0.01 mol L^{-1} phosphoric acid 30:70 was used as mobile phase at flow rate 0.5 mL min^{-1}. Injection volume was 5 (µl of the sample. Also LC Alliance Systems (Waters) with diode array detector was used with the same column and gradient elution of 0% to 100% methanol in mixture with 0.1% trichlorocetic acid in 25 min.

LC-MS system consisted of HP 1100 Series LC system (Hewlett-Packard) and MS ion trap detector with electrospray ionisation (Finnigan, DECA). LC-MS analyses were carried out at 20°C on the Phenomenex Luna C18 column (150 x 2 mm, 3 µm) with guard column Cl8 (4 x 2 mm). The mobile phase was a mixture of water (A) and acetonitrile (B). The gradient was 5-100% B in 40 min at flow rate of 0.2 mL min^{-1}.

For quantification of phenolic compounds photometric signals at selected wavelengths measured on HPLC system Dionex with Chromeleon software were chosen. Analyses were carried out at ambient temperature on a glass column (150 x 3.2 mm) packed with Separon SGX Cl8 , 7 µm (Tessek) with mixture of methanol - 0,01 mol L^{-1} phosphoric acid 30:70 as mobile phase at flow rate 0.5 mL min^{-1}. Sample injection volume was 5 µL. In this system the separation of 3,4-dihydroxybenzoic acid, p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, ferulic acid, benzoic acid and *p*- hydroxyacetophenone was possible.

Extraction procedures

Three procedures were used in this study to isolate UV-light screening substances from Norway Spruce needles.

1. *Hot Water extraction.* 50 g sample was mixed with 400 mL of water boiled in a round bottom flask under reflux for 1 hour. Samples were cooled to room temperature; aqueous layer was separated, centrifuged, filtered and analysed by HPLC.
2. *Methanolic extraction.* Needles (about 2.7 g) were cut with a pair of scissors, then extracted three times in an ultrasonic bath (Bandelin Sonorex) for 45 minutes with 8 mL of 80% methanol in water. Combined extracts were collected and the volume was adjusted to 25 mL. The supematant was centrifuged, filtered and analysed by HPLC.
3. Acid hydrolysis. Aliquots of water and methanolic extracts were filtered and mixed with 6 mol L-l HCI. Final solution had concentration of 1.7 mol L-l. The solution was kept at temperature 70 °C on water bath for 2 hours. Then solution was cooled, centrifuged and the supematant analysed by HPLC.

Results and discussion

Compounds which were identified in methanolic and aqueous extracts using LC with diode array and mass spectrometric (MS) detection are listed in Table I.

Substances found (+) in aqueous and methanolic extracts by LC with DAD and MS detection.

Table I

Phenolic Compound	DAD detection		MS detection	
	Aqueous Extract	Methanolic Extract	Aqueous Extract	Methanolic Extract
p-Hydroxyacetophenone	+	+	+	+
Catechin	+	+	+	+
p-Hydroxybenzoic acid	-	+	+	+
Vanillic acid	-	-	+	+
3,4-Dihydroxybenzoic acid	-	-	+	+
Benzoic acid	-	-	+	+
Ferulic acid	-	-	+	+
p-Coumaric acid	+	+	+	-

Commercially available preparations of these compounds have the same retention times as well as absorption and MS spectra as the peaks observed in the chromatograms of extracts. When extracts were analysed by LC-MS, it was possible to identify more compounds than with diode array detection because better separation of weakly retained components was achieved with gradient elution.

A lot of compounds observable in chromatograms of the needle extracts could not be identified. Some of them have mutually similar absorption spectra and hence may contain the same chromophore. According to mass spectra some of these unidentified peaks display a large relative mass. These facts suggest that these compounds could be esters or conjugates of phenolic compounds with sugar units. It is known that phenolic compounds frequently occur in plants in such forms. [4]

In order to decompose the conjugates, the extracts were treated by acid hydrolysis. After hydrolysis new peaks appeared in chromatograms, but we were not able to identify them. Amount of other components originally present in extracts increased. However, catechin and other components disappeared in LC chromatograms. Only traces of catechin were still detected by LC-MS.

In the next step we tried to quantify the studied compounds in extracts before and after hydrolysis. As aqueous and methanolic extracts were prepared from different batches of samples, the results are not fully comparable. These assays were carried out on the LC system Dionex with photometric detection. Separations for detection wavelength 310 nm are shown in Fig. 2. It was possible to quantify only *p*- hydroxybenzoic acid, p-hydroxyacetophenone, p-coumaric, ferulic and benzoic acids on various preselected detection wavelengths. The other peaks present in the chromatograms were not quantified because of poor resolution from other components or lack of identification. Results of determination are summarised in Table II.

Table II

Amount of substances (μg g^{-1} of needles) determined in aqueous and methanolic extracts before and after acid hydrolysis

Phenolic Compound	Before Hydrolysis		After Hydrolysis	
	Aqueous Extract	Methanolic Extract	Aqueous Extract	Methanolic Extract
p-Hydroxyacetophenone	44	165	565	1498
Catechin	-	-	-	-
p-Hydroxybenzoic acid	-	+	45	56
Vanillic acid	-	-	-	-
3,4-Dihydroxybenzoic acid	-	-	-	-
Benzoic acid	5.3	-	-	-
Ferulic acid	-	63	12	-
p-Coumaric acid	12	67	36	102

In most cases higher amounts of components are found after hydrolysis. In aqueous extract p-coumaric acid originally present at a concentration of 12 μg g^{-1} increased to 36 μg g^{-1} after hydrolysis and ferulic acid appeared in the concentration of 12 μg^{-1} The results for

methanolic extract are similar, p-coumaric acid at 67 µg g^{-1} in the original extract increased after hydrolysis to 102 µg g^{-1}. The resolution of components on the chromatograms did not enable to quantify more compounds. However, it is evident that free phenolic compounds are released from other components of the extract by acid hydrolysis. However, some phenolic compounds like catechin may decompose by this treatment.

Because extracts from Norway Spruce needles are complex mixtures, which are difficult to separate by single LC analysis, we attempted to find a pre-separation method suitable for sample clean up. Preferable would be a method separating components to groups according to their type, as such methods may aid in the component identification. We studied two following LC systems, which appear promising in their ability to separate phenols and phenolic acids.

Separation based on the ion exclusion principle

This type of separation is usually carried out on the ion exchangers carrying the same charge as separated compounds. Because of ionic repulsion, the porous structure of the sorbent is not accessible to species of the same charge. It is possible to separate in this way acids on cation exchangers.

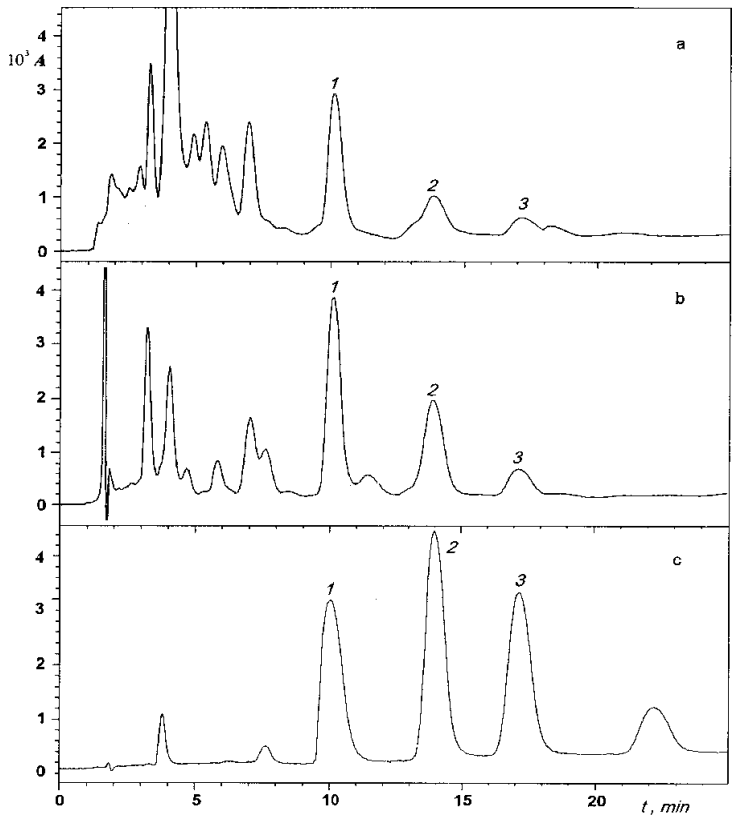

Fig.2

Chromatogram (X =310 nm) of aqueous extract of Norway spruce needles (a), the same extract after acid hydrolysis (b) and mixture of standards (c)

1- p-Hydroxyacetophenone, 2 - p-Coumaric acid, *3* - Ferulic acid.
HPLC system Dionex with Chromeleon software, glass column 150 x 3.2 mm Separon SGX Cl8, 7 µm, mobile phase methanol - 0.01 mol L^{-1} phosphoric acid 30:70, 0,5 mL min^{-1}, photometric detection at 310 nm

Common silica-based reversed-phase sorbents usually contain residual silanol groups which are weakly acidic and can operate in this manner. Otherwise, a similar effect can be induced by the sorption of an anionic detergent added to the mobile phase. We had added sodium dodecylsulfate (SDS) to the mobile phase and studied the retention behaviour of some phenolic acids and neutral compounds on the Separon SGX C-18 column. The mobile phase consisted of methanol - aqueous SDS of concentration in the range 0.01 to 30 mmol L^{-1} (Fig. 3).

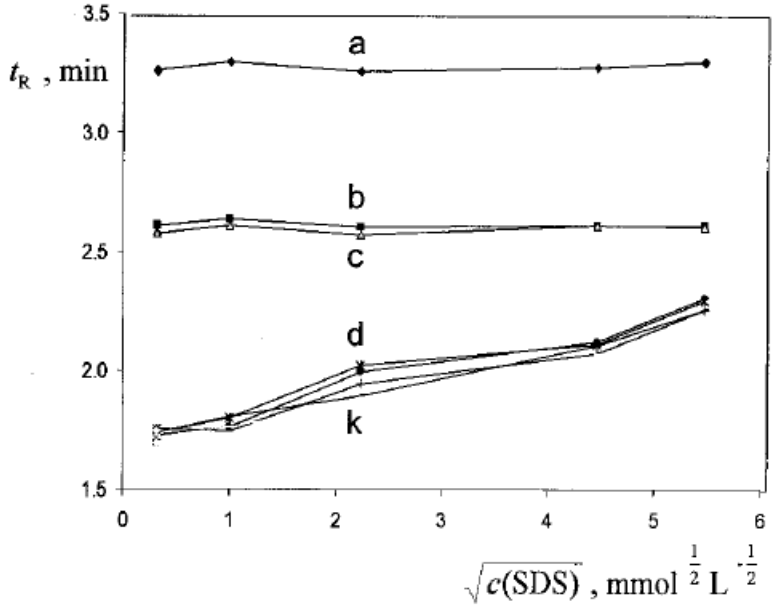

Fig. 3
Retention of phenolic compound at ion-exclusion conditions

Column 150 x 3.2 mm, Separon SGX C18, 7 µm, mobile phase methanol-aqueous SDS c(SDS) = 0.1 to 30 mmol L^{-1}, flow rate 0.4 mL min^{-1}.

p-Hydroxyacetophenone (a), Phloroglucinol (b), Catechin (c), p-Hydroxybenzoic acid (d), p-Cinnamic acid (e), Vanillic acid (f), p-Coumaric acid (g), Protocatechuic acid (h), Benzoic acid (i), Ferulic acid (j), m-Coumaric acid (k)

In these experiments phloroglucinol was used as a dead volume marker. Due to the exclusion of phenolic acids from the pores of the sorbent, they are eluted from the column before the dead time. From the graph it is possible to conclude that this separation system enables, in principle, the fractionation of phenolic acids and compounds that do not possess a carboxylic group like p-hydroxyacetophenone and catechin. SDS concentration of about 0.1 mmol L^{-1} in the aqueous component of the mobile phase is adequate for this separation.

Table III
Retention behaviour of phenolic compounds and alcohols under hydrophilic interaction conditions. Column Lichrosorb Si-60, 7 µm, 150 x 3.2 mm, mobile phase acetonitrile- water, 0.4 mL min^{-1}

Compound	Retention Time, min				
	Acetonitrile – Water				
	50:50	80:20	95:5	98:2	100:0
p-Hydroxybenzoic acid	1.27	1.58	2.81	8.59	>15
p-Coumaric acid	1.22	1.53	3.01	9.57	>15
p-Ferulic acid	-	-	-	10.88	-
Vanillic acid	-	-	-	12.52	-
Benzoic acid	-	-	8.4	9.94	>15
Quercetin	>15	-	>15	-	>15
Rutin	>15	-	>15	-	>15
Catechin	1.82	1.92	2.09	-	>15
p-Hydroxyacetophenone	1.83	1.89	2.04	2.10	2.30
Phenol	1.88	-	1.98	-	2.05
Phloroglucinol	1.86	-	2.05	-	2.23
Pyrocatechol	2.03	-	2.17	-	2.35
Resorcinol	1.86	-	1.99	-	2.11
Hydroquinone	1.96	-	2.02	-	2.17
Glycerol	1.98	-	-	-	-
Ethylene glycol	1.94	-	3.66	-	7.58
Benzyl alcohol	1.93	-	2.00	-	2.33

It is not possible, however, to apply the method directly to samples of high ionic strength like that obtained after hydrolysis of extracts, because high content of ions interferes with the principle of ion exclusion.

Separation based on the hydrophilic interactions principle

The term hydrophilic interactions denote interactions between strongly polar functional groups, which play important role in LC separations of polar compounds on polar sorbents. Very simple separation system of this type is represented by silica gel with acetonitrile as the mobile phase. We have studied retention behaviour of phenolic acids, phenols and other hydroxy-compounds on LiChrosorb Si-60 column with mixtures acetonitrile-water as a mobile phase. From the retention data in Table III follows the possibility to separate acids from other phenolic compounds with pure acetonitrile as the mobile phase. Also ethyleneglycol and

glycerol display higher retention than phenolic compounds which are not retained on the column. Only rutin and quercetin is retained very strongly and could not be eluted even with mobile phases with high water content. For this type of pre-separation, however the sample must be dissolved in acetonitrile because even low amount of water added to the mobile phase readily decreases the retention of all compounds (again except for rutin and quercetin).

Conclusions

In extracts from Norway spruce needles besides p-hydroxyacetophenone as major component and catechin, six phenolic acids were identified. A large number of components remain unidentified. Some phenolic compounds are extracted also in the form of conjugates, probably glycosides or esters, and may be released by acid hydrolysis. However, catechin is broken down by hydrolysis.

In order to simplify chromatograms and eliminate interfering components, sample fractionation based on ion-exclusion or hydrophilic interactions is promising and will be a subject of further study.

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