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Content of phenolic compounds in meadow vegetation and soil depending of the isolation method

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Received: date; Accepted: date; Published: date

Abstract: The aim of this paper was to determine the effect of the hydrolysis method on the amount of phenolic compounds in the plant material in soil and, as a consequence, on the parameters to determine the degree of lignins transformation in soils. The study included the plant material samples (hay, sward and roots) and soils - Albic Brunic Arenosol (horizon A, AE and Bsv). Phenolic compounds were at two stages by applying the acidic hydrolysis followed by alkaline re-hydrolysis. The quantitative and qualitative analysis of phenolic compounds was performed with High Performance Liquid Chromatography with the DAD detector. The content of phenolic compounds in the extracts depended on the hydrolysis method and it was determined by the type of the research material. The amount of phenolic compounds contained in the alkaline hydrolysates accounted for 55.7 (soil, horizon Bsv) – 454% (roots) of their content in acid hydrolysates. In the extracts from acid hydrolysates, chlorogenic and *p*-hydroxybenzoic acids were dominant. In the alkaline extracts from plant material, the highest content was recorded for *p*-coumaric and ferulic acids, and in the extracts from soil – ferulic and chlorogenic acids. A combination of acid and alkaline hydrolysis ensures the best extraction efficiency of insoluble-bound forms of polyphenols from plant and soil material.

Keywords: meadow soils; acids hydrolysis; alkaline hydrolysis; phenolic compounds; HPLC

1. Introduction

Phenolic acids and phenolic aldehydes represent a group of polyphenols, common in plant tissues. They are secondary metabolites with a varied chemical composition and biological properties [1,2]. In plants they are mostly found in bound forms, as esters or glycosides, as lignin and tannins hydrolyzing components [1]. As for the chemical composition, phenolic acids, as lignin derivatives, present in plant cells, can be divided into groups which differ in the number of and the place of substitution of hydroxyl and methoxyl groups:

–hydroxybenzoic acid and its derivatives, e.g. dihydroxybenzoic, protocatechuic (PA), salicylic (SA), syringic (SYR), vanillic acids ~~(VA)~~;

– hydroxycinnamic acid and its derivatives, e.g. cinnamic, ferulic (FEA), *p*-coumaric (*p*-CO), caffeic acids ~~(CA)~~.

Another group of phenolic compounds are depsides the molecule core of which includes an ester bond, e.g., chlorogenic acid (CHA) is produced as a result of the combination of the carboxyl group of caffeic acid with phenolic group of quinic acid [2].

Phenolic compounds can be isolated from solid samples using many different methods, e.g., simple extraction, extraction combined with hydrolysis, ultrasound or microwave-radiation-assisted extraction [1, 3-4]. Very often the simple extraction of phenolic compounds using organic solvents shows a low performance as it mostly allows for isolating free phenolic compounds and disregarding

the bound forms. To isolate the insoluble-bound forms, it becomes justifiable to apply preliminary hydrolysis, followed by extraction [1,3].

Verma et al. [4] and Irakli et al. [5] report on the use of the Folin-Ciocalteu reagent to facilitate assaying the total values of both free and bound phenolic acids. Verma et al. [4] and Ross et al. [6] report on isolating phenolic acids from the plant material samples with the selection of the type of the hydrolysis applied (acid, alkaline) being essential. Verma et al. [4] have demonstrated that higher contents of *p*-coumaric, vanilic and sinapic acids were recorded as a result of the alkaline hydrolysis, as compared with the contents of the compounds produced from the acid hydrolysis, whereas, as for syringic acid (SYR), an inverse dependence was recorded. Besides, the authors show that some phenolic compounds are soluble in the acid environment and some – in the alkaline one.

Phenolic compounds isolating and assaying in plant material are also covered by Ross et al. [6]. They compare three sample hydrolysis methods: alkaline hydrolysis followed by acid hydrolysis; alkaline hydrolysis alone and acid hydrolysis alone. Each method comes in two variants: the first one involved adding ascorbic acid (AA) and ethylenediaminetetraacetic acid (EDTA), while in the second variant – no solutions of those acids were added. As a result, the highest performance was noted after the application of alkaline hydrolysis with AA and EDTA, as compounds protecting phenolic compounds against decomposition, added. The effect of adding AA and EDTA on the stability of phenolic compounds has also been confirmed by Nardini et al. [7]. The results reported by Verma et al. [4] and Ross et al. [6] demonstrate that, to receive the total contents of phenolic acids and aldehydes found in the research material sample, both types of hydrolysis should be applied and AA and EDTA should be added.

The papers which report on a comparison of the hydrolysis method for the plant material and soils are missing. Assaying the contents of phenolic compounds in soils facilitates the evaluation of the degree of plant residue decomposition in soil [8,9,10,11,12,13]. Besides, determining at least the mutual proportions of phenolic compounds of syringyl, vanillyl and cinnamyl type gives a possibility of determining the genus of the plant undergoing decomposition in soil [11,12,14,15].

The aim of this paper was to determine the effect of the hydrolysis method on the amount of phenolic compounds recorded in the plant material (hay - H, sward - St, root - R) and soils (horizons A, AE and Bsv) and, as a result, on the parameters, to determine the degree of lignins transformation in soils.

2. Results

In the extracts from acid hydrolysates and alkaline re-hydrolysates from the plant material and soil samples, 11 phenolic aldehydes and phenolic acids have been identified (**Table S1**).

There was a considerable variation in the content of respective phenolic compounds which occur in the extracts from acid hydrolysates and alkaline re-hydrolysates and the plant material and soil samples. Sample chromatograms for separating the phenolic compounds contained in the extracts after acid hydrolysis and alkaline re-hydrolysis are presented in **Figure 1**.

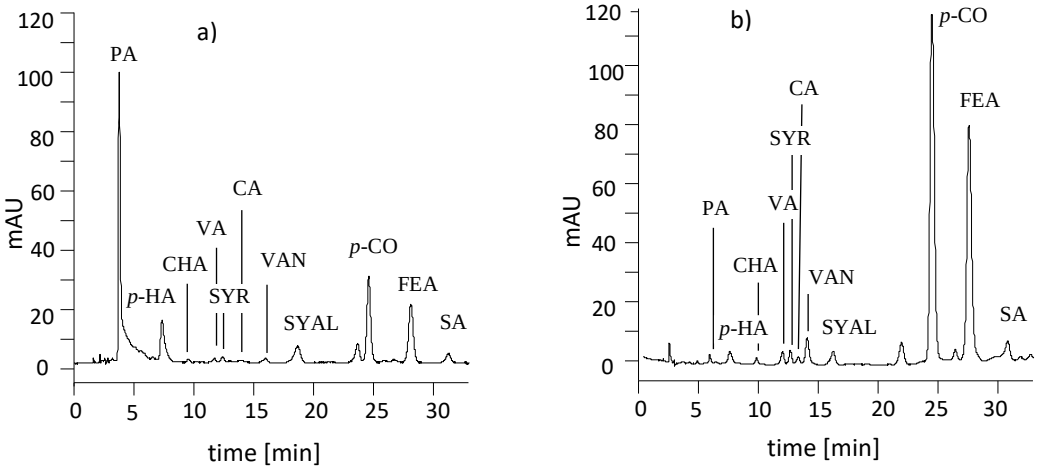


Figure 1. Selected RP-HPLC chromatogram of phenolic compounds of the extracts from hay: **a** - following acid hydrolysis, **b** - following alkaline re-hydrolysis.

2.1. Contents of phenolic compounds in the plant material samples

The quantitative analysis of the phenolics of the hydrolysates from the plant material has shown that in the extracts from alkaline re-hydrolysates the significantly higher contents of the total phenolic compounds analysed were recorded, as compared with their contents in the extracts from acid hydrolysates (**Table 1**). The average contents of the total aldehydes and phenolic acids in the extracts from alkaline re-hydrolysates were: 4900.3 (hay), 3598.5 (sward) and 2585.2 μg/g d.w. (roots), which accounted for: 75.9, 81.0 and 84.7% of the phenolic compounds isolated as a result of both types of hydrolysis. The extracts from acid hydrolysates showed higher contents of *p*-hydroxybenzoic acid (*p*-HA) than the extracts from alkaline re-hydrolysates. Besides, from the hay and sward samples, with the method of acid hydrolysis, more chlorogenic acid (CHA) was produced than after alkaline re-hydrolysis. CHA in the extracts after acid hydrolysis accounted for 84.4 and 68.9% of its total from both hydrolyses. In the extracts from acid hydrolysates from the root samples, 70.7 μg/g d.w. less CHA was assayed than in the extracts from alkaline re-hydrolysates.

98 **Table 1.** Content of phenolic compounds in plant material [μg/g d.w.].

Symbol	mean ±SD	min	max	SE	mean ±SD	min	max	SE
	acid hydrolysis				alkaline re-hydrolysis			
				Hay				
PA	73.0 ±62.8[a]	20.2	230.8	14.8	74.9 ±19.6[a]	47.9	107.1	4.6
p-HA	72.2 ±51.8[b]	13.8	182.5	12.2	59.9 ±19.7[a]	19.4	79.3	4.7
CHA	710.8 ±151.6[b]	530.0	947.4	35.7	130.9 ±29.9[a]	90.0	183.1	7.0
VA	49.6 ±17.2[a]	23.9	79.3	4.0	110.7 ±24.2[b]	83.0	158.2	5.7
SYR	42.5 ±21.2[a]	18.8	86.1	5.0	99.2 ±29.5[b]	70.2	156.9	6.9
CA	64.2 ±41.3[a]	13.4	147.8	9.7	166.6 ±33.9[b]	105.5	228.3	8.0
VAN	76.4 ±39.3[a]	24.2	149.7	9.3	261.4 ±68.9[b]	179.5	385.6	16.2
SYAL	60.8 ±32.4[a]	18.5	114.2	7.6	248.2 ±48.9[b]	180.1	328.1	11.5
p-CO	145.9 ±24.5[a]	105.9	189.7	5.8	1655.1 ±304.7[b]	1275.0	2221.0	71.8
FEA	201.7 ±41.7[a]	137.1	256.1	9.8	1988.0 ±452.4[b]	1306.9	2500.4	106.6
SA	58.9 ±32.6[a]	14.8	116.7	7.7	106.0 ±61.2[b]	20.5	214.1	14.4
SUM	1556.1 ±153.0[a]	1272.0	1816.7	36.1	4900.9 ±685.7[b]	3900.2	6083.7	161.6
				Sward				
PA	55.1 ±26.1[a]	12.1	91.5	6.2	77.3 ±20.2[b]	56.8	111.2	4.8
p-HA	90.6 ±84.3[b]	18.3	278.4	19.9	78.6 ±38.5[a]	33.6	143.8	9.1
CHA	315.3 ±151.6[b]	126.5	638.6	35.7	142.6 ±33.8[a]	100.5	202.5	8.0
VA	42.5 ±32.5[a]	9.2	112.1	7.7	116.1 ±24.4[b]	73.8	148.6	5.8
SYR	37.9 ±23.5[a]	10.0	84.6	5.5	73.0 ±20.1[b]	33.8	102.1	4.7
CA	77.9 ±42.7[a]	36.9	164.8	10.1	127.4 ±27.4[b]	77.0	165.1	6.5
VAN	44.0 ±17.2[a]	17.3	73.8	4.1	201.2 ±68.0[b]	117.9	348.1	16.0
SYAL	60.0 ±28.0[a]	30.5	107.4	6.6	134.9 ±53.6[b]	40.6	219.0	12.6
p-CO	47.1 ±34.5[a]	7.3	107.3	8.1	1426.2 ±253.5[b]	1005.8	1738.4	59.8
FEA	58.9 ±30.1[a]	17.8	114.5	7.1	1092.2 ±220.0[b]	900.6	1585.4	51.8
SA	12.8 ±7.7[a]	2.8	25.7	1.8	129.0 ±88.7[b]	42.8	263.2	20.9
SUM	842.2 ±347.7[a]	507.0	1594.0	82.0	3598.5 ±477.8[b]	3048.3	4592.5	112.6
				Roots				
PA	33.6 ±13.3[a]	10.9	56.1	3.1	71.6 ±26.2[b]	22.5	105.9	6.2
p-HA	121.8 ±13.7[b]	92.6	140.9	3.2	100.2 ±38.4[a]	34.0	161.8	9.1
CHA	81.7 ±41.9[a]	15.1	134.0	9.9	152.4 ±30.4[b]	115.3	211.7	7.2
VA	16.2 ±8.3[a]	3.3	30.4	2.0	118.2 ±28.0[b]	57.2	149.8	6.6
SYR	20.6 ±6.7[a]	9.7	32.0	1.6	82.1 ±48.0[b]	26.0	164.0	11.3
CA	71.7 ±38.1[a]	23.3	146.3	9.0	154.1 ±38.9[b]	100.9	238.9	9.2
VAN	22.4 ±9.7[a]	9.8	37.4	2.3	112.6 ±15.0[b]	90.3	139.6	3.5
SYAL	40.0 ±18.5[a]	10.9	71.4	4.4	96.7 ±45.8[b]	20.0	165.6	10.8
p-CO	17.4 ±13.9[a]	4.0	45.1	3.3	891.6 ±214.2[b]	477.8	1128.6	50.5
FEA	31.6 ±22.7[a]	3.4	65.7	5.3	732.5 ±63.8[b]	629.2	843.7	15.0
SA	9.6 ±2.0[a]	6.8	13.3	0.5	73.2 ±21.0[b]	46.2	114.8	4.9
SUM	466.6 ±81.8[a]	367.0	586.9	19.3	2585.2 ±210.4[b]	2175.0	2883.9	49.6

99 a, b – mean values with the same letter are not significantly different at 5 %

The mean contents of the other phenolic compounds, in the extracts from alkaline re-hydrolysates, were significantly higher, as compared with their mean contents in the extracts from acid hydrolysates. The biggest differences in terms of the mean content of the phenolic compounds analysed were recorded for *p*-coumaric acid (*p*-CO). The differences were 1509.2, 1379.1 and 874.2 μg/g d.w. for H, St and R, respectively. When applying the acid hydrolysis, there was isolated only 8.1, 3.2 and 1.9% of the total content of *p*-CO. With the alkaline re-hydrolysis method there were also recorded high contents of ferulic acid (FEA), which for H, St and R were, on average, 1988.0, 1092.2 and 732.5 μg/g d.w. and accounted for 90.8, 94.9 and 95.9% of the content of that acid in both hydrolysates. The differences in the content of vanillic aldehyde (VAN) in the H, St and R samples across extracts were 185.0, 157.2 and 90.2 μg/g d.w. The method of acid hydrolysis allowed only for assaying 22.6, 17.9 and 16.6% of the total contents of VAN, respectively. The contents of salicylic acid (SA) in the alkaline re-hydrolysates from hay, sward and roots were 106.0, 129.0 and 73.2 μg/g d.w., which accounted for 64.3, 91.0 and 88.4% of the content of that acid assayed with the two methods. The extracts from alkaline re-hydrolysates contained 248.2, 134.9 and 96.7 μg/g d.w. of syringic aldehyde (SYAL), which accounted for 80.3, 69.2 and 70.7% of the total contents of that aldehyde. The differences in the contents of caffeic acid (CA) across the extracts of hay, sward and roots were 102.4, 49.5 and 82.4 μg/g d.w. The method of alkaline re-hydrolysis allowed for assaying 72.2, 62.1 and 68.2% of the total amounts of CA produced as a result of both hydrolyses. The share of vanillic acid (VA) in the extracts from alkaline re-hydrolysates from hay, sward and roots accounted for 69.1, 73.2 and 87.9% of the total VA contents.

2.2. Contents of phenolic compounds in soil samples

The extracts from acid hydrolysates and alkaline re-hydrolysates of the soil sampled 5 and 25 m away from the irrigation ditch from three horizons (A, AE and Bsv) demonstrated a considerable variation in the mean contents of phenolic compounds (**Table 2**). The total contents of all the phenolic aldehydes and phenolic acids analysed in the extracts from alkaline re-hydrolysates of the samples of soil from horizon A were, on average, 310.4 μg/g d.w. higher than in the extracts from acid hydrolysates. The alkaline re-hydrolysis method facilitated assaying 70.6% of the total amounts of phenolic compounds isolated in those samples applying both hydrolyses. Only the mean contents of vanillic (VA), caffeic (CA) acids and both aldehydes (VAN, SYAL) in the extracts from acid hydrolysates and alkaline re-hydrolysates were similar. The contents of the other compounds, namely *p*-CO, SA, FEA, PA, CHA, *p*-HA and SYR, in the extracts from alkaline re-hydrolysates accounted for 96.4, 83.8, 79.1, 76.0, 75.4, 69.6 and 65.7% of the total of both types of hydrolyses, respectively.

134 **Table 2.** Content of phenolic compounds in plant material and soil [μg/g d.w.].

Symbol	mean ±SD	min	max	SE	mean ±SD	min	max	SE
	acid hydrolysis				alkaline re-hydrolysis			
				horizon A				
PA	7.6 ±2.3[a]	3.6	11.8	0.5	24.1 ±10.2[b]	8.1	44.0	2.4
p-HA	10.9 ±5.3[a]	4.1	21.1	1.2	25.0 ±15.7[b]	10.0	56.6	3.7
CHA	39.3 ±30.3[a]	12.1	104.3	7.1	121 ±62.2[b]	23.9	215.0	14.7
VA	26.4 ±6.8[a]	17.8	40.2	1.6	30.2 ±12.2[a]	10.5	50.9	2.9
SYR	13.7 ±4.0[a]	9.8	23.9	0.9	26.2 ±9.7[b]	10.4	42.8	2.3
CA	42.0 ±21.8[a]	23.6	90.1	5.1	33.4 ±13.6[a]	10.3	54.0	3.2
VAN	17.8 ±12.0[a]	6.0	43.7	2.8	19.1 ±10.7[a]	9.0	40.4	2.5
SYAL	25.9 ±11.5[a]	8.1	49.9	2.7	32.1 ±14.9[a]	10.6	57.1	3.5
p-CO	3.1 ±2.6[a]	0.8	8.7	0.6	84.1 ±42.8[b]	15.1	144.3	10.1
FEA	29.9 ±9.0[a]	17.2	46.0	2.1	113.3 ±46.4[b]	30.3	177.6	10.9
SA	4.5 ±3.4[a]	2.0	13.5	0.8	23.2 ±16.2[b]	4.6	49.3	3.8
SUM	221.2 ±44.5[a]	166.5	307.6	10.5	531.6 ±132.3[b]	334.9	770.9	31.2
				horizon AE				
PA	16.3 ±7.7[b]	6.8	30.4	1.8	7.2 ±4.2[a]	3.0	17.9	1.0
p-HA	77.0 ±27.8[b]	40.4	126.1	6.6	10.9 ±4.7[a]	5.1	21.3	1.1
CHA	54.1 ±24.6[a]	21.2	90.5	5.8	49.5 ±24.8[a]	10.0	79.0	5.8
VA	17.3 ±9.3[b]	6.1	34.0	2.2	12.0 ±8.6[a]	3.2	29.4	2.0
SYR	16.4 ±14.0[a]	2.0	43.5	3.3	13.1 ±5.5[a]	3.3	23.1	1.3
CA	35.8 ±19.9[a]	6.1	66.2	4.7	28.8 ±16.0[a]	7.2	55.2	3.8
VAN	17.5 ±11.3[a]	3.2	34.5	2.7	12.9 ±6.6[a]	4.0	27.0	1.6
SYAL	19.7 ±12.3[b]	9.2	47.2	2.9	13.6 ±12.5[a]	3.6	44.4	2.9
p-CO	4.3 ±4.0[a]	0.4	13.9	0.9	11.4 ±7.7[b]	4.7	29.1	1.8
FEA	9.4 ±3.7[a]	2.1	13.4	0.9	50.2 ±14.1[b]	30.2	77.3	3.3
SA	5.4 ±3.9[a]	1.8	15.9	0.9	12.3 ±6.8[b]	6.3	28.8	1.6
SUM	273.1 ±50.2[b]	187.2	355.7	11.8	221.9 ±47.9[a]	148.7	296.0	11.3
				horizon Bsv				
PA	13.3 ±7.6[b]	3.3	29.9	1.8	8.3 ±2.6 [a]	5.3	13.4	0.6
p-HA	57.5 ±29.2[b]	12.9	92.3	6.9	12.9 ±5.4[a]	5.1	24.8	1.3
CHA	44.4 ±21.4[b]	18.2	86.7	5.0	21.6 ±7.4[a]	11.1	35.0	1.7
VA	14.6 ±9.0[b]	6.3	34.7	2.1	8.7 ±6.1[a]	4.0	25.0	1.4
SYR	12.6 ±3.2[b]	6.4	17.2	0.7	6.4 ±3.7[a]	2.0	13.8	0.9
CA	27.5 ±18.9[b]	8.4	60.0	4.5	8.6 ±4.6[a]	3.3	17.2	1.1
VAN	12.9 ±6.1[b]	6.5	24.4	1.4	4.6 ±1.6[a]	2.4	8.0	0.4
SYAL	13.4 ±15.1[b]	1.6	48.6	3.6	6.6 ±1.5[a]	3.7	9.6	0.3
p-CO	4.3 ±2.4[a]	1.4	9.3	0.6	9.5 ±6.8[b]	2.0	22.7	1.6
FEA	12.2 ±8.6[a]	3.3	30.7	2.0	22.6 ±15.3[b]	7.8	58.7	3.6
SA	3.6 ±2.2[a]	1.1	6.8	0.5	10.8 ±9.2[b]	3.0	33.0	2.2
SUM	216.5 ±30.2[b]	154.5	262.6	7.1	120.5 ±29.7[a]	75.8	184.1	7.0

135 a, b – mean values with the same letter are not significantly different at 5 %

In the soil sampled from eluvial horizon (AE) and illuvial (Bsv) horizon the total content of phenolic compounds in the extracts from acid hydrolysates, as compared with the alkaline re-hydrolysates, was higher. The differences were 51.2 (AE) and 96.0 μg/g d.w. (Bsv), respectively. With the acid hydrolysis there were recorded 55.2 and 64.2% of the total phenolic compounds produced as a result of both hydrolyses, respectively.

For the soil sampled from horizon AE, the highest difference across the extracts was recorded for *p*-hydroxybenzoic acid (*p*-HA). The share of *p*-HA extracted with the method of acid hydrolysis accounted for 87.6% of its total content recorded applying both methods. The extracts from acid hydrolysates showed also higher contents of protocatechuic (PA), vanillic (VA) acids and syringic aldehyde (SYAL) as compared with the content in the extracts from alkaline re-hydrolysates. There were demonstrated no significant differences in the content of chlorogenic acid (CHA), syringic acid (SYR), caffeic acid (CA) and vanillic aldehyde (VAN) between acid hydrolysis and alkaline re-hydrolysis. The content of ferulic (FEA), *p*-coumaric (*p*-CO) and salicylic (SA) acids was higher in the solutions after alkaline re-hydrolysis than in the solutions after acid hydrolysis. The share of those compounds in alkaline re-hydrolysates accounted for 84.2, 72.6 and 69.5% of the total of those compounds produced as a result of both hydrolyses, respectively.

For the soil sampled from the illuvial horizon (Bsv) in the extracts of acid hydrolysates, *p*-hydroxybenzoic acid (*p*-HA) was identified most. Its content in acid hydrolysates accounted for 81.7% of the total of both hydrolyses. In the extracts studied, the lowest difference in the content was noted for protocatechuic acid (PA). Its share in acid hydrolysates accounted for 61.6% of the total from both hydrolyses. The method of alkaline re-hydrolysis, on the other hand, facilitated isolating 75.0, 68.8 and 64.9% of the total amounts of salicylic (SA), *p*-coumaric (*p*-CO) and ferulic (FEA) acids.

3. Discussion

The contents of phenolic compounds recorded in the samples of plant and soil material have unambiguously demonstrated that, additionally, the application of alkaline hydrolysis after acid hydrolysis considerably increased the amount of the phenolic compounds extracted. As stressed earlier by Mroz et al. [1], Verma et al. [4], Ross et al. [6], Nardini et al. [7], Krygier et al. [16] the importance of the hydrolysis method selected on the quality and the amount of the phenolic compounds was reported.

The literature most frequently presents the results of research on the application of the acid hydrolysis method to isolate phenolic compounds from the samples of plant material and soils [1,6,13,17,18]. Verma et al. [4], however, note the importance of application of the method of alkaline re-hydrolysis after acid hydrolysis in terms of the amount of the phenolic compounds received. After alkaline re-hydrolysis, Verma et al. (2009) [4], Kim et al. [19] recorded even a few fold higher amounts of ferulic and *p*-coumaric acids, as compared with the amounts assayed in the samples after acid hydrolysis alone. Also, the other identified phenolic compounds point to a possibility of producing their higher amounts, which was confirmed in the research presented in this paper.

For all the samples of plant material (hay, sward, roots) and soil sampled from horizon A, there have been recorded higher total contents of phenolic compounds, as a result of alkaline re-hydrolysis, as compared with acid hydrolysis alone (**Table 1 and 2**). Interestingly, for the plant material and soil of horizon A, the alkaline re-hydrolysis method provided the grounds for producing ferulic (FEA) and p-coumaric (*p*-CO) acids and, as for sward, roots and soil of horizon A – also for salicylic acid (SA). The share of FEA, *p*-CO and SA in alkaline re-hydrolysates of horizons AE and Bsv was also higher than in the acid hydrolysates. Verma et al. [4] report on the method of acid hydrolysis facilitating a release of considerably higher amounts of salicylic acid than alkaline re-hydrolysis. However, due to the differences in the research material, and mostly the chemical composition of the materials compared, one cannot expect that the dependencies will be identical.

In the acid hydrolysates of the root and soil sampled from horizons AE and Bsv, *p*-hydroxybenzoic acid was dominant. The samples of hay and sward, on the other hand, provided considerably higher amounts of *p*-hydroxybenzoic and chlorogenic acids, as compared with their amounts isolated with the alkaline re-hydrolysis method.

A significantly higher share of phenolic compounds in the extracts after acid hydrolysis, as compared with alkaline hydrolysis for the soil sampled from horizons AE and Bsv, can be a consequence, next to the tissue structure and a decreasing amount of organic matter of plant origin, deep down the soil profile, of a change in the proportions of the compounds of soil organic matter. The higher the degree of organic matter humification, the higher the share of hydrolyzing compounds in the acid solution, as compared with the compounds hydrolyzing in the alkaline solution. In horizon A, the presence of the extended meadow vegetation root system [21] affects the amount of organic matter undergoing decomposition, and thus the content of phenolic compounds, especially those isolated in the alkaline reaction solution.

To sum up, it must be stressed that, to avoid a risk of underestimating the content of phenolic compounds which occur in the material studied, if possible, two types of hydrolysis should be applied.

Isolating and identifying phenolic compounds allow for dividing those compounds into three groups referred to as:

- vanillyl compounds (V), as the total content of vanillin and vanillic acid derived from coniferyl alcohol;
- syringyl compounds (S), as the total content of syringaldehyde and syringic acid, derived from sinapyl alcohol;
- cinnamyl compounds (C), as the total content of ferulic acid, *p*-coumaric acid and caffeic acid, derived from coumaryl alcohol.

The ratios of those compounds in lignins are plant-species-specific. It was also shown that, despite a considerable transformation of lignins during plant material humification, determining the ratio of the compounds allows for determining their origin [8,9,12,13,14,15]. As reported by Kovaleva and Kovalev [12], lignins of herbaceous plants contain higher amounts of cinnamyl compounds than the contents of vanillyl and syringyl compounds. The lignins of grasses and meadow herbs can contain from 4- to 6-fold more cinnamyl compounds than the lignins of trees, and the V:S:C ratio for the aboveground part of grass vegetation of sub-Alpine meadows was 1:1:6. As reported by e.g. Crawford [20], angiosperms lignins contain approximately equal amounts of vanillyl and syringyl compounds and little cinnamyl compounds. Gymnosperms lignins show a definite advantage of vanillyl compounds over the other ones.

In this paper the content of vanillyl, cinnamyl and syringyl compounds and their share in the V+S+C pool have been calculated to verify the effect of the method of isolating phenolic compounds on the parameters studied. There have been compared the parameters calculated for the contents of phenolic compounds in acid hydrolysates and for the sum of both hydrolyses (acid hydrolysis + alkaline hydrolysis = sum (total) hydrolyses).

Irrespective of the methods of assaying the phenolic compounds (acid hydrolysis, sum of both hydrolyses) applied, the contents of vanillyl, syringyl and cinnamyl compounds were definitely higher in the plant material, as compared with their contents in soil. Interestingly, both for the plant material samples and for the soil samples, no significant differences in the content of vanillyl and syringyl compounds were found, whereas cinnamyl compounds were dominant.

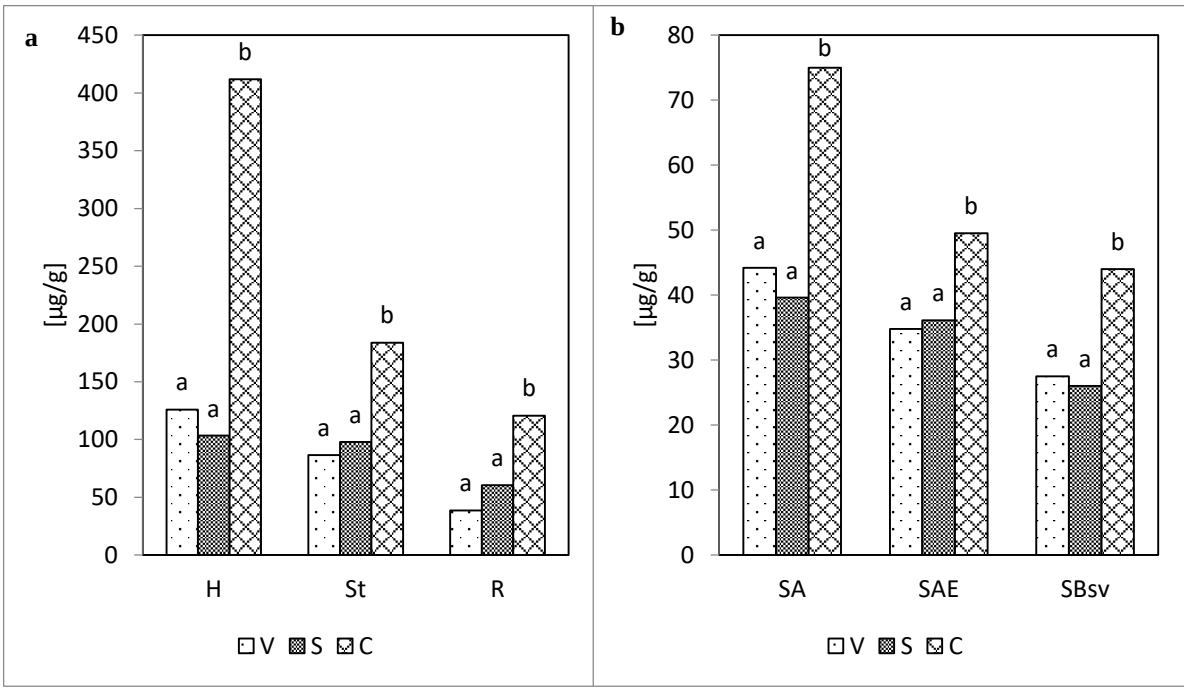


Figure 2. Content of vanillyl (V), syringyl (S) and cinnamyl (C) compounds following acid hydrolysis: **a** – in the plant material (H, St, R), **b** – in soil (SA, SAE, SBsv).

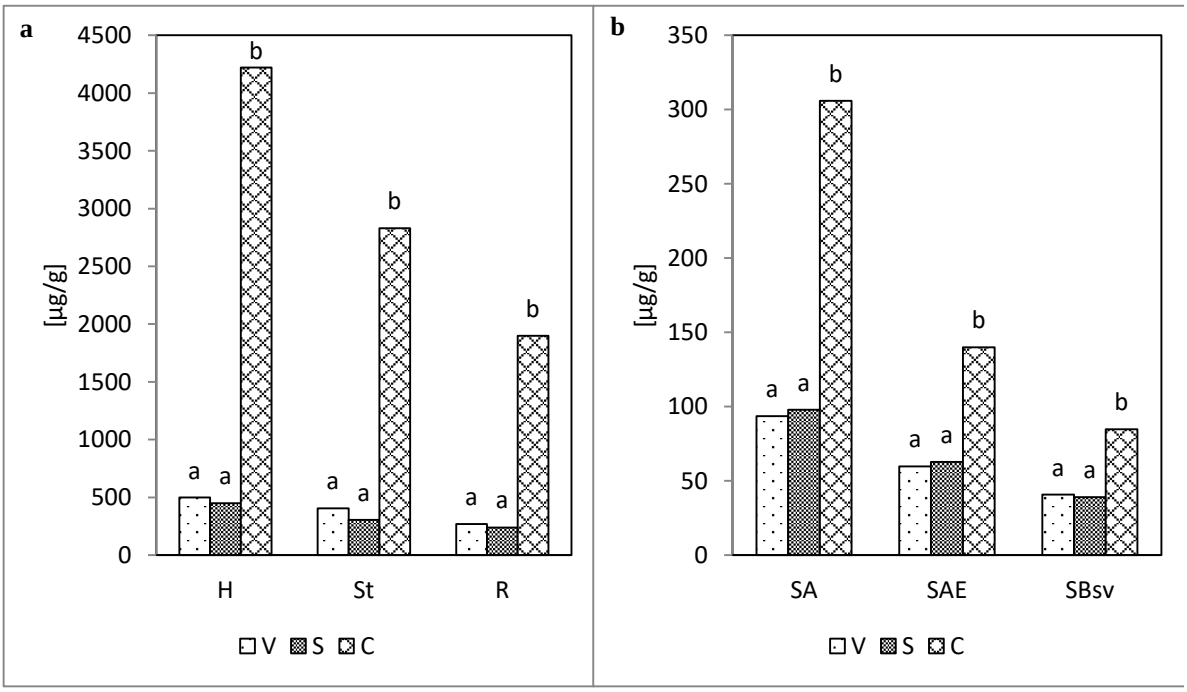


Figure 3. Content of vanillyl (V), syringyl (S) and cinnamyl (C) compounds following both hydrolyses (acid and alkaline): **a** – in the plant material (H, St, R), **b** – in soil (SA, SAE, SBsv).

The share of cinnamyl compounds in the V+S+C pool (**Figure 4**), following the application of both hydrolyses in plant material (hay, sward, roots), ranged from 78.87 to 81.65 %, and in soil – from 51.49 to 61.5 %, whereas after the application of acid hydrolysis only, the share of cinnamyl compounds accounted for 49.93 – 64.24 % and 41.12 – 47.23 %, respectively (**Figure 4**). It is obviously a consequence of a significant effect of the method of alkaline hydrolysis on the efficiency of the extraction of *p*-coumaric and ferulic acids.

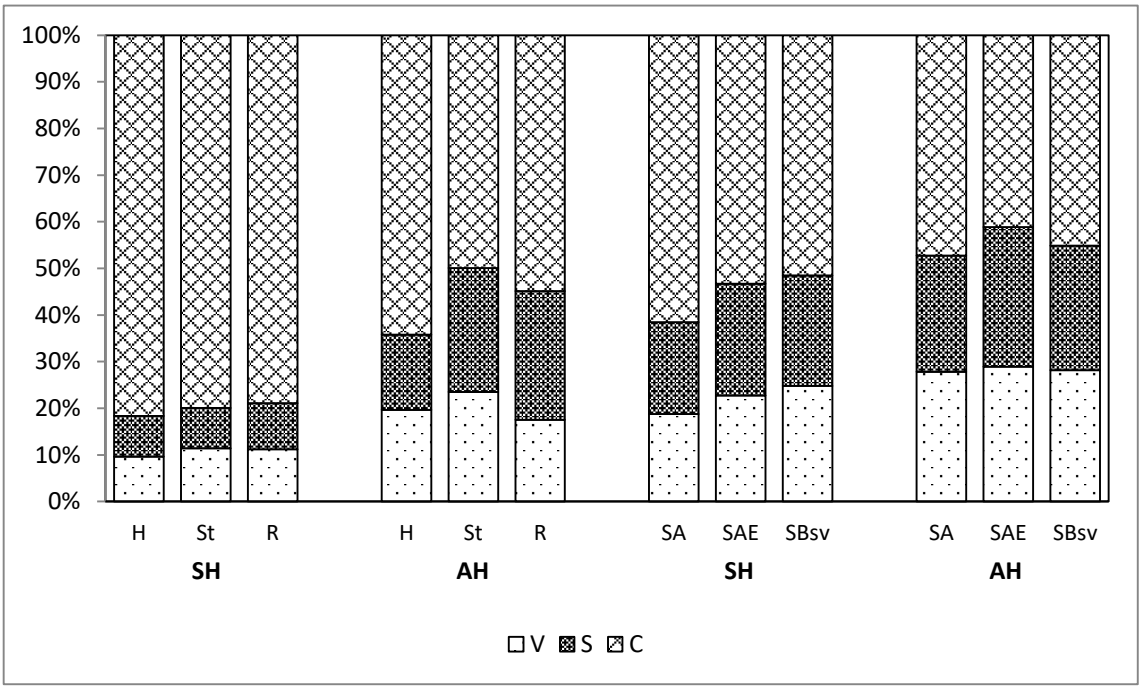


Figure 4. Share of vanillyl (V), syringyl (S) and cinnamyl (C) compounds in V+S+C, following acid hydrolysis (**AH**) and both hydrolyses (**SH**, acid and alkaline).

As seen from **Table 3**, irrespective of the method applied, the ratio of vanillyl to syringyl compounds was, in general, 1:1. The V:S:C parameter, following the application of both hydrolyses for plant material, ranged from 1:1:8 to 1:1:9 and for soil – from 1:1:2 to 1:1:3, whereas, after the application of acid hydrolysis only, it was 1:1:2-1:1:4 and 1:1:1.5-1:1:2, respectively. However, it must be stressed that, irrespective of the method of determining the contents of phenolic compounds, one records the dependencies which allow formulating identical conclusions for the changes which occur in the process of plant material humification in soils.

Table 3. Ratio of the share of vanillyl (V), syringyl (S) and cinnamyl (C) compounds – V:S:C in hydrolysates of plant material (H, St, R) and soil (SA, SAE, SBsv).

Material	Sum of hydrolyses	Acids hydrolysis
H	1:1:9	1:1:4
St	1:1:9	1:1:2
R	1:1:8	1:1.5:3
SA	1:1:3	1:1:2
SAE	1:1:2.5	1:1:1,5
SBsv	1:1:2	1:1:1.5

4. Materials and Methods

4.1. Chemicals

HCl, NaOH, ascorbic acid, EDTA, ethyl acetate~~: p.a., provided by Avantor Performance Materials, Poland.~~

~~Phenolic compounds, analytical standard: protocatechuic acid, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, vanillin, ferulic acid, salicylic acid provided by Sigma-Aldrich; vanillic acid, syringic acid, syringaldehyde, *p*-coumaric acid provided by Fluka~~

~~Acetonitrile, methanol, acetic acid, HPLC purity, provided by Avantor Performance Materials, Poland.~~

The water was purified using the Millipore Milli-Q system.

4.2. Materials

The samples were taken in the country's and Europe's unique perennial grasslands complex, the Czerskie Meadows (53°87′ N; 18°12′ E). The Czerskie Meadows Complex was established in the mid 19th century and it is located in various parts of the Tuchola Forest (Poland). In total it covers the area of about 2000 ha. The uniqueness of that complex is related to the irrigation system, namely the slope-and-flood system applied back then, which, in most areas of the Czerskie Meadows, has still survived.

The samples were taken from the plot where irrigation had been stopped since 1996, referred to by the founders as the Green Meadow. The plant material (hay, sward and roots) and soil (horizons A, AE and Bsv) were sampled from three specified stands. At each stand the soil was sampled 5 and 25 m away from the irrigation ditch. The soil was sampled from 6 soil profiles (a total of 18 plant material and 18 soil samples). The soil samples were dried in room temperature and sieved (2 mm). The plant material samples were dried in room temperature and ground. There were analysed 18 plant material samples and 18 soil samples, whereas the results provided in the tables are mean values for the three stands specified and two distances (5 and 25 m). The soil in all the profiles was analysed in compliance with the WRB systematics as *Brunic Albic Arenosol*. The basic soil properties are presented in **Table 4** [21].

Table 4. Basic parameters of soils and grain size composition (mm).

Horizon		TOC (g/kg)	Nt (g/kg)	pH	0.2-0.05	0.05-0.002 (%)	<0.002
A	Mean	39.72	3.65	-	91.74	8.25	n.d.**
	Min	30.20	2.80	5.2	89.45	6.68	-
	Max	45.20	4.15	6.5	93.32	10.53	-
	SE*	6.00	0.49	-	1.29	1.28	-
AE	Mean	28.73	2.77	-	91.33	9.20	n.d.
	Min	21.40	2.13	5.6	88.70	6.19	-
	Max	35.60	3.41	6.7	93.81	11.82	-
	SE	6.31	0.57	-	1.83	2.21	-
Bsv	Mean	15.83	1.44	-	89.30	10.20	0.02
	Min	10.00	0.47	5.9	85.72	8.58	0.02
	Max	24.00	2.49	6.7	91.42	13.28	0.02
	SE	6.63	0.84	-	2.19	1.90	-

* - standard error, ** - not determined, TOC – total organic carbon, Nt – total nitrogen

4.3. Methods

4.3.1. Extraction of phenolic compounds from the samples of plant material and soils

To isolate the phenolic compounds from the plant material and soils, two types of hydrolysis have been applied:

–acid hydrolysis with a HCl solution;

–alkaline re-hydrolysis ~~performed after acid hydrolysis~~ (**Figure 5**).

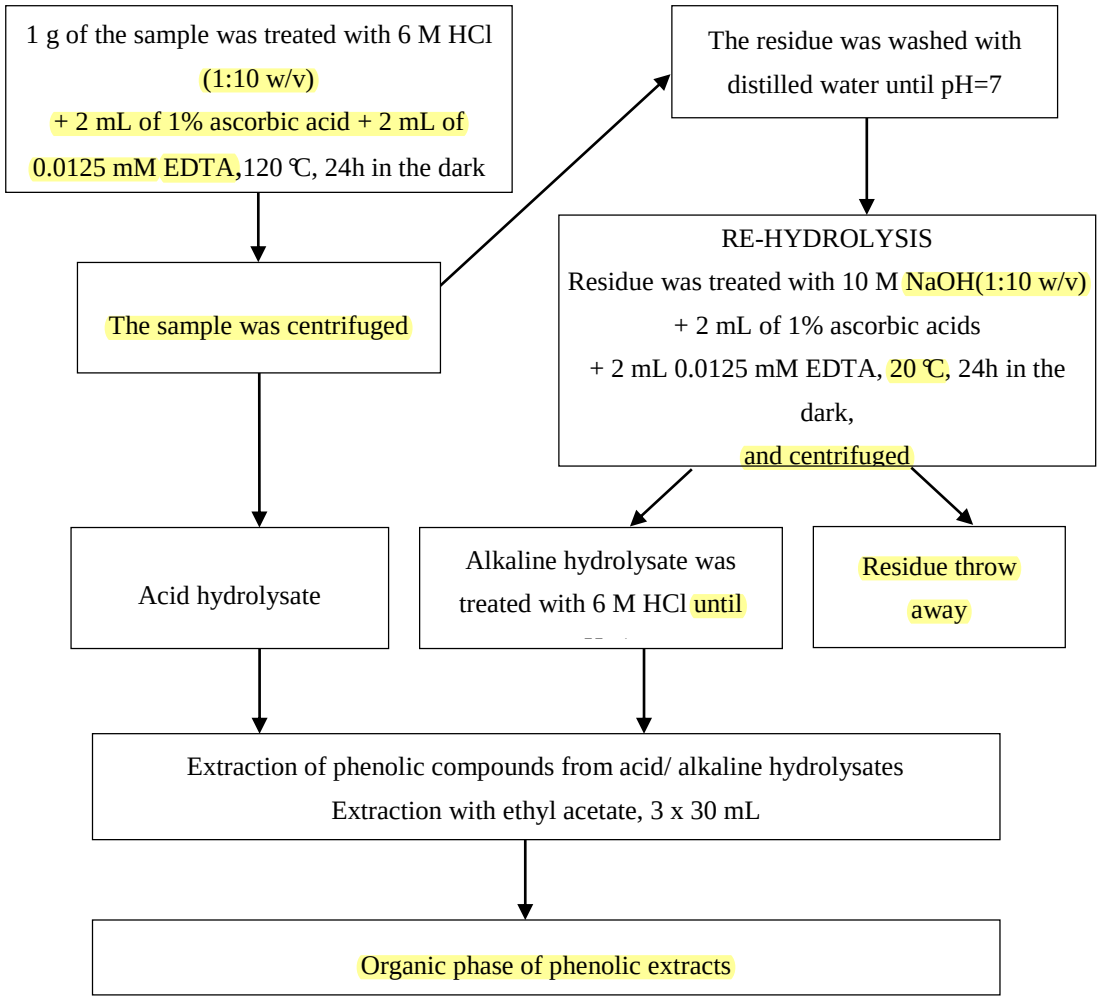


Figure 5. Extraction procedure for phenolic compounds from plant material and soil (modification of the methods described by Verma et al. 2009 and Ross et al. 2009)

4.3.2. Qualitative and quantitative analyses of extracts containing phenolic compounds

The organic phase of phenolic extracts was evaporated until dry and then dissolved in 2.5 mL MeOH. The qualitative and quantitative composition of phenolic compounds contained in the extracts from hydrolysates from the samples of the plant material; the samples of soils were assayed using the high-performance liquid chromatographer HPLC Series 200 by Perkin–Elmer (Shelton, USA) equipped with the DAD detector. Analytic column Bionacom Velocity STR with 5 μm in particle diameter and 250 × 4.6 mm in size was used.

The mobile phase consisted of:

- eluent A: H_2O:CH_3CN:CH_3COOH (88.5:10:1.5 – % V),
- eluent B: CH_3CN.

The samples for analysis were prepared by combining 0.5 mL filtered (nylon filter, φ=0.45 μm) of the extract of phenolic compounds with 0.5 mL of eluent A. The injection volume was 10 μL ~~(three replications)~~. The gradient division program was applied at the rate flow of 1.3 mL/min. The initial composition of the mobile phase was 100% of eluent A applied. The content of eluent B was growing linearly from the 14th minute of the analysis and it reached 10% in 36 minutes, then decreased, reaching at the final stage of the analysis, namely in the 42nd minute, 0%. The detection was made at the wavelength of 280 nm. The pattern of the chromatogram for the reference solution of phenolic compounds and their retention times (Figure 6) facilitated identifying the following phenolic compounds: protocatechuic acid (PA), *p*-hydroxybenzoic acid (*p*-HA), chlorogenic acid (CHA),

vanillic acid (VA), syringic acid (SYR), caffeic acid (CA), vanillin (VAN), syringaldehyde (SYAL), *p*-coumaric acid (*p*-CO), ferulic acid (FEA) and salicylic acid (SA) (Table S1). The content of phenolic compounds is given in μg/g d.-w. The quantitative analysis of the phenolic compounds identified was performed using the external reference curves of the dependence of the peak area for the concentration of phenolic compound (μg/mL). The reference response curve for each phenolic compound was a linear regression with correlation coefficients > 0.98. The HPLC analysis of reference solutions was performed as for the phenolic extracts.

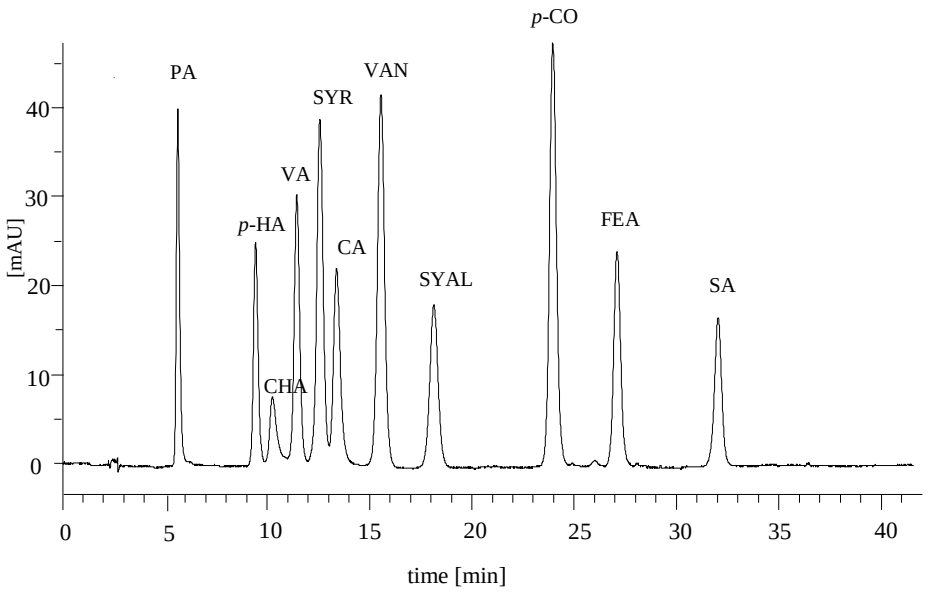


Figure 5. Phenolic compounds reference mixture chromatogram

4.4. Statistical analysis

The results were statistically verified by determining the following: mean, maximum and minimum, standard deviation (SD) and standard error (SE). The significance of the differences between the content of vanillyl, syringyl and cinnamyl compounds for each variant (hay, sward, roots, soil: horizon A, AE and Bsv) was determined with the Kruskal-Wallis test, the median test and the U Mann-Whitney test, at the level of significance of p=0.05. For statistical calculations, the Excel spreadsheet and Statistica MS 2012 package were applied.

5. Conclusions

1. A combination of acid and alkaline hydrolysis ensures the best extraction efficiency of insoluble-bound forms of polyphenols from plant and soil material.
2. Alkaline re-hydrolysis has resulted in an increase in the amount of the phenolics extracted from the plant material samples from 215% (hay) to 454% (roots), and from soil – from 55.7% (horizon Bsv) to 240% (horizon A) of their contents in acid hydrolysates.
3. In the extracts from acid hydrolysates, in general, chlorogenic and *p*-hydroxybenzoic acids were dominant. In the alkaline extracts from the plant material, the highest content was recorded for *p*-coumaric and ferulic acids, and in the extracts from soil – for ferulic and chlorogenic acids.
4. The values of parameter V:S:C (the ratio of vanillyl to syringyl to cinnamyl compounds) recorded for the extracts from the plant material following the acid hydrolysis ranged from 1:1:2 to 1:1:4, and for the hydrolyses sum – from 1:1:8 to 1:1:9. The values of parameter V:S:C

for soil, calculated for the hydrolyses in total were 1:1:2 – 1:1:3, and following the acid hydrolysis only – 1:1:1.5 – 1:1:2.

5. It has been demonstrated that the plant material of meadow soils, irrespective of the extraction method, showed a similar share of syringyl and vanillyl compounds and an advantage of cinnamyl compounds. In the humification process of meadow vegetation the ratio of the share of V and S compounds does not change, however, the share of cinnamyl compounds in the V+S+C pool decreases.

Author Contributions: Conceptualization, A.Z., B.D. and M.B-Sz.; Methodology, A.Z., B.D. and M.B-Sz.; Investigation, A.Z. and B.D.; Data curation – compiled and analysed the results, A.Z., B.D. and M.B-Sz.; Writing – original draft preparation, A.Z., B.D. and M.B-Sz.; Writing – review and editing, A.Z., B.D. and M.B-Sz. All the authors have read and agreed to the published version of the manuscript

Acknowledgments: The authors with to thank the University of Science and Technology in Bydgoszcz for supporting this research (part of BS-3/2017 research project).

Conflicts of Interest: The authors declare no conflict of interest.

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