Development of a rapid resolution HPLC method for the separation and determination of 17 phenolic compounds in crude plant extracts

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Abstract: Rapid Resolution HPLC/DAD method, on a 1.8 μm, 4.6×50 mm column, was developed to enable a rapid separation of a mixture of 17 compounds, which consisted of hydroxybenzoic acids, hydroxycinnamic acids, flavones, flavonols, flavanone, flavonol-glycoside and antraquinone, in a single run, within 22 minutes. The developed method is precise, accurate and sensitive enough for simultaneous quantitative evaluation of major compounds in crude and hydrolyzed extracts of parsley, buckthorn, mint, caraway and birch. In order to overcome the inability to quantify all the phenolic compounds present in the samples caused by lack of external standards, HPLC approaches for the total phenolic content estimation based on sum of all integrated peak areas were made. These results were compared with the total phenolic content determined by Folin–Ciocalteu method. Although the correlation between the series of data was not significant (p<0.05), the difference between the results of total phenolic content obtained spectrophotometrically and by HPLC was not high in the case of parsley, buckthorn and mint extract. Regarding the obtained results, the HPLC approach could serve as an excellent tool for total phenolic content estimation, without the need for complete identification of the individual compounds.

Keywords: Plant phenolics • Rapid Resolution HPLC • Medicinal plant

1. Introduction

Crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. Determination of the amounts and species of polyphenols in crude extracts is one of the greatest challenges for analysts because of the number of natural phenolics belonging to different classes, which has been estimated to be over one million. These compounds are found in plants in the form of glycosides, differing greatly in number, kind and bonding type of their sugar moieties [1,2].

The most widely used method for determination of absolute concentrations of complex phenolic mixtures in plant extracts, the Folin-Ciocalteu method, is based on the reduction of a phosphotungstic-phosphomolybdic reagent in slightly alkaline medium [3]. The Folin–Ciocalteu assay for total phenols is affected by several interfering substances such as sulfur dioxide, ascorbic acid, sugar, aromatic amines, organic acids, Fe(II) and other nonphenolic organic substances that react with the reagent [4].

On the other hand, large number of instrumental chromatography methods for separation and determination of phenolic compounds in plant matrices has been published. These methods are usually adjusted either for determination of the most represented phenolics in certain plant species, or for determination of certain number of phenolic compounds in various matrices [5].

Referring to the review article by Molnár-Perl et al. [6], high performance liquid chromatography (HPLC) occupies a leading position in the analysis of...
phenolic composition. In general, HPLC separations are based on C18 reverse-phased columns and a binary solvent gradient. The mobile phase usually consists of an aqueous solution of acid and an organic solvent (acetonitrile or methanol). The number of analytes detected simultaneously by most HPLC methods is usually around 10, which are usually satisfactorily separated within 45 or 50 min.

Concerning flavonoid content in plants, papers describing methods which cover analysis of five classes of flavonoids have been published [7,8]. In both cases, quantification was done after hydrolysis of glycosides, by comparing the results with available external standards of aglycones. Merken and Beecher [7] have developed an HPLC system for the separation and quantification of seventeen flavonoids, using 5 μm particle reversed–phase (C18) column. Although most of the flavonoids had peak-to-peak separation of 0.5 min or greater, myricetin and pelargonidin as well as kaempferol and apigenin were only partially resolved, within the relatively long total run time of 60 min. Harnly et al. [5] has determined 20 flavonoids in fresh fruits, vegetables, and nuts, by using gradient elution with methanol, acetonitrile and trifluoroacetic acid during a 60 min run.

Sakakibara et al. [8] has developed an HPLC/DAD method for simultaneous determination of all phenolic substances in foodstuffs. By using 5 μm C18 column, a separation of simple polyphenols, catechins, anthocyanins, glycosides of flavones, flavonols, isoflavones and flavanones, their aglycons, anthraquinones, chalcones, and theaflavins was achieved, but the total run time of 95 min was required to achieve the elution.

Due to a wide range of phenolics present in plant extracts, there is a need for a method which allows simultaneous detection of a wide range of phenolics in a single run, in order to decrease the time necessary for the analysis of complex mixtures and reduce the analysis costs. This method should be able to characterize the occurrence of flavonoids and phenolic acids in various materials with sufficient selectivity and sensitivity during a short analysis time based on efficient separation. However, recent methods often show weak points in one or more of these requirements. Furthermore, due to its toxicity and shortage on the market, use of acetonitrile or methanol, acetonitrile and trifluoroacetic acid during a 60 min run.

In order to reduce the analysis time and maintain good efficiency in liquid chromatography, high temperature liquid chromatography (up to 90°C) can be applied. Raising the temperature (from 80 to 200°C) can induce a 5- to 10-fold reduction in the mobile phase viscosity [9], allowing the application of high flow rates with limited back-pressure. However, compound stability needs to be evaluated prior to analysis.

Regarding the Van Deemter theory, sub-2-micron particle packed columns have enhanced sensitivity and separation power in comparison to the conventional and monolithic columns, which results in decreased analysis time and solvent consumption [9]. The use of sub-2-micron particles may lead to column back-pressures of more than 60 MPa. Today, numerous manufacturers commercialize analytical devices that are able to handle pressure higher than 400 bar, such as UPLC (Ultra Performance Liquid Chromatography) and Rapid Resolution HPLC. In order to lower a column back-pressure, higher temperatures can be applied, which can influence the stability of thermolabile compounds.

Regarding all the above mentioned, we decided to develop an Rapid Resolution HPLC method suitable for determination of different phenolic compounds in crude extracts of selected medicinal herbs, on a short C18 analytical column packed with 1.8 μm silica-based particles, using methanol as an organic solvent in a binary mobile phase system. Also, in order not to affect the sample composition caused by possible thermal degradation of the compounds, we decided not to apply the elevated temperatures during the analyses.

Therefore, the aim of this study was to develop a rapid, sensitive and selective method for the quantitative analysis of a mixture of 17 compounds, which consisted of hydroxybenzoic acids, hydroxycinnamic acids, flavones, flavonols, flavanone, flavonol-glycoside and anthraquinone in selected medicinal herbs.

2. Experimental Procedure

2.1 Chemicals and materials
Methanol (HPLC, gradient grade), quercetin and formic acid (HPLC) were supplied by Merck KGaA (Darmstadt, Germany). Folin-Ciocalteau’s reagent and standard substances including gallic acid, protocatechuic acid, caffeic acid, vanillic acid, chlorogenic acid, syringic acid, ferulic acid, rutin, myricetin, rosmarinic acid, trans-cinnamic acid, naringenin, luteolin, kaempferol, apigenin and aloe-emodin were purchased from Sigma-Aldrich GmbH (Sternheim, Germany). Water used throughout the experiments was purified using a Millipore, Elix UV and Simplicity Water Purification System (Milford, MA, USA).

Herbal drugs: parsley (Petroselinum fructus), buckthorn (Frangulae cortex), mint (Mentha piperitae folium), caraway (Carvi fructus) and birch (Betulae folium) were obtained from the Institute for Medicinal Plants Research “Dr Josif Pančić” from Belgrade.
2.2 Sample preparation
Crude plant extracts were obtained by maceration with ethanol/water mixture (80:20, v/v), with the ratio of raw materials to ethanol solution of 1:10, for 24 h at room temperature and subsequently extracted in an ultrasonic bath at room temperature for 10 min. Mass of the extracts was measured after filtration through a filter paper (Whatman, Grade 4 Chr, UK) and vacuum-evaporation of the solvent. Hydrolysis of extracts was performed as described by Justesen et al. [10]. All samples were redissolved in a mixture of methanol:1% formic acid in water (1:1, v/v), to obtain a final extract concentration of 8.00-15.00 mg mL⁻¹. Prior to injection, the sample solutions were filtered through a 0.20 μm membrane PTFE filter (Rotilabo-Spritzenfilter 13 mm, Roth, Karlsruhe, Germany).

2.3 Spectrophotometric determination of total phenolics
Total phenolics were determined spectrophotometrically by using Folin-Ciocalteu’s reagent and the results are expressed as gallic acid equivalents [4]. Gallic acid calibration curve (c = 97.1070×A + 0.0101, R² = 0.9984) was plotted on the basis of seven calibration points within the range of 1.00 – 6.00 µg mL⁻¹ of gallic acid in the reaction mixture.

2.4 Instrumentation and analytical conditions
HPLC analysis was performed by using a liquid chromatograph (Agilent 1200 series), equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies), a binary pump, an online vacuum degasser, an autosampler and a thermostated column compartment, on an Agilent, Eclipse XDB-C18, 1.8 µm, 4.6×50 mm column, at a flow-rate of 1 mL min⁻¹. Solvent gradient was performed by varying the proportion of solvent A (methanol) to solvent B (1% formic acid in water (v/v)) as follows: initial 10% A; 0-10 min, 10 -25% A; 10-20 min, 25 - 60% A; 20-30 min, 60-70% A. The total running time and post-running time were 45 and 10 min, respectively. The column temperature was 30ºC. The injected volume of samples and standards was 5 μL and it was done automatically using autosampler. The spectra were acquired in the range 210–400 nm and chromatograms plotted at 280, 330 and 350 nm, with a bandwidth of 4 nm, and with reference wavelength/bandwidth of 500/100 nm.

2.5 Statistical analysis
The experimental results were given as mean ± SD of at least three parallel extractions and measurements. Statistical data analysis software system STATISTICA (StatSoft, Inc. (2008). data analysis software system, version 8.0., www.statsoft.com) was used for analysis. P values < 0.05 were regarded as significant.

3. Results and Discussion
3.1 Performance of the chromatographic method
Reversed-phase liquid chromatography using acetonitrile gradient under acidic mobile phase conditions is a common practice in the separation of complex samples [11]. Because of the potential toxicity of acetonitrile and shortage on the market, we decided to develop a method using methanol instead of acetonitrile.

Chromatogram of external standards mixture is presented in the Figs. 1A-1C recorded at 280 nm, 330 nm and 350 nm, respectively. As can be seen from the chromatogram, all of the investigated compounds had the responses at 280 nm, where the components were successfully separated, with the exception of quercetin and naringenin. We have overcome that problem by determining them at 330 nm, where these compounds gave the responses as two separated peaks. Identification of phenolic compounds in crude extracts and hydrolisates was performed by comparing the retention times and spectra of phenolic compounds of extracts with those of the corresponding external standards. Quantification was based on external standards calibration. Stock solutions of individual standards were prepared at the concentration of 1.00 mg mL⁻¹ in methanol. The solutions were properly diluted with 1% formic acid to obtain a series of dilutions in the range of 0.005-34.00 µg mL⁻¹ in methanol: 1% formic acid in water (1:1, v/v) for external standard calibration and linearity check at 280, 330 and 350 nm, with the exception of quercetin and naringenin, which were tested only at 330 nm, and phenolic acids and aloe-emodin which had the responses only at 280 nm. The regression equation and linearity range for each compound, together with LOD and LOQ values and recoveries are shown in Table 1. Calibration curves were plotted on the basis of at least five calibration points and the correlation coefficients were calculated. Strong linear relationships were obtained for all investigated compounds (R² > 0.9995) at the wavelengths where the compounds had the responses, with the exception of quercetin and naringenin, which were tested only at 330 nm. Despite the fact that quercetin and naringenin peaks were resolved at 330 nm, they were not fully separated (resolution of 0.601 was obtained at the concentration of 20.00 µg mL⁻¹). However, quantitation...
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Figure 1. Chromatogram of external standard mixture, absorbance at A-280 nm, B-330 nm and C-350 nm: 1-gallic acid, 2-protocatechuic acid, 3-cafeic acid, 4-vanillic acid, 5-chlorogenic acid, 6-syringic acid, 7-ferulic acid, 8-rutin, 9-myricetin, 10-rosmarinic acid, 11-trans-cinnamic acid, 12-quercetin, 13-naringenin, 14-luteolin, 15-kaempferol, 16-apigenin and 17-alo-emodin.
of the compounds was successfully achieved, as the mixture of standards was used for the calibration for the samples that contained both compounds. By keeping the integration events for quercetin and naringenin constant throughout the experiment, high regression coefficients for the calibration curves and recovery values were obtained (Table 1). The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively. Although the slope of gallic acid calibration curve is higher than that of protocatechuic acid, based on S/N ratio, LOD value of gallic acid was estimated to be higher due to the higher noise of the baseline in the region of gallic acid.

Since the purpose of this work was not to test the efficiency of the extraction method, the recovery was determined by spiking the investigated medicinal plant extracts with the mixture of all external standards, each of the standards at a mass concentration of 0.50 µg mL⁻¹. The content of investigated compounds was previously determined in non spiked medicinal plant extracts. For each phenolic compound, the recovery was calculated on the basis of the ratio of the obtained amount in the spiked extract to the calculated amount (sum of the amounts originated from extract and standard).

Retention time and peak area were checked for repeatability by injecting the mixture of standards at the concentration of 0.01 mg mL⁻¹ into the HPLC system over six runs. The within- and inter-day relative standard deviation (RSD) of the retention times was less than 0.20% for all standards, and less than 3.00% for the obtained peak area.

Regarding the results shown in Table 1, the developed method is precise, accurate and sensitive enough for simultaneous quantitative evaluation of major compounds in crude and hydrolyzed extracts of parsley, buckthorn, mint, caraway and birch. Chromatogram of the crude birch extract prior to and after hydrolysis is presented in the Figs. 2A, 2B, respectively.

### 3.2 Sample analyses

All samples were extracted and analysed in triplicate. Content of investigated plant phenolic compounds in crude extracts and in crude extracts after hydrolysis is expressed as mg g⁻¹ extract as a mean value ± SD (Tables 2 and 3). Despite the good injection precision, the results of repeatability, as indicated by SD values, exhibited more variation. An explanation for the fluctuations in replicate analyses is the degree of sample (in)homogeneity and also the noticeable amount of essential oil present in dried plant material which could impair sample wettability and consequently the extraction [11].

Large number of papers concerning determination of phenolic content of birch, parsley, mint, caraway, as well as anthraquinone content of buckthorn has been published [8,12-15].

In crude ethanolic extract of mint, the following compounds were identified and quantified: caffeic acid, chlorogenic acid, rutin, rosmarinic acid, quercetin, naringenin, luteolin and apigenin (Table 2). In comparison to other quantified compounds, rosmarinic acid was the most abundant. After hydrolysis, increase in the concentration of all compounds occurred due to the growth of the microorganisms present in the plant material. In crude ethanolic extract of mint, the following compounds were identified and quantified: caffeic acid, chlorogenic acid, rutin, rosmarinic acid, quercetin, naringenin, luteolin and apigenin (Table 2). In comparison to other quantified compounds, rosmarinic acid was the most abundant. After hydrolysis, increase in the concentration of all compounds occurred due to the growth of the microorganisms present in the plant material.

### Table 1. Calibration curves, linearity ranges, LODs, LOQs and recoveries of external standards.

| Compound               | Calibration curve | Regression coefficient \((R^2)\) | Linear range \((\mu g \ mL^{-1})\) | \(\lambda\) \(\text{(nm)}\) | LOD \((\mu g \ mL^{-1})\) | LOQ \((\mu g \ mL^{-1})\) | Recovery \(\text{Mean}\) (%) | RSD (%) |
|------------------------|-------------------|----------------------------------|-----------------------------------|----------------|----------------|----------------|----------------|---------|
| gallic acid            | \(y = 14252.5x + 0.487\) | 0.9999                           | 0.060-17.0                        | 280            | 0.018          | 0.060          | 109.0          | 1.4     |
| protocatechuic acid    | \(y = 7412.3x + 0.156\)  | 1.0000                           | 0.010-23.0                        | 280            | 0.003          | 0.010          | 87.8           | 2.5     |
| caffeic acid           | \(y = 11239.0x - 10.526\)  | 0.9995                           | 0.010-34.0                        | 330            | 0.063          | 0.211          | 102.0          | 3.1     |
| vanillic acid          | \(y = 6436.2x - 5.550\)   | 0.995                            | 0.007-30.0                        | 280            | 0.055          | 0.182          | 92.0           | 2.4     |
| chlorogenic acid       | \(y = 35165.0 - 19.700\)   | 0.9995                           | 0.006-26.2                        | 330            | 0.009          | 0.032          | 109.0          | 4.3     |
| syringic acid          | \(y = 15375.9x + 0.654\)   | 1.0000                           | 0.010-22.0                        | 280            | 0.010          | 0.032          | 129.0          | 3.2     |
| ferulic acid           | \(y = 14096.3x - 0.848\)   | 0.9999                           | 0.010-22.0                        | 280            | 0.058          | 0.195          | 132.0          | 4.2     |
| rutin                  | \(y = 3646.4x - 0.085\)    | 0.9999                           | 0.009-17.0                        | 280            | 0.009          | 0.039          | 96.2           | 5.2     |
| myricetin              | \(y = 11374.3x - 0.435\)   | 0.9999                           | 0.005-11.0                        | 350            | 0.003          | 0.009          | 81.0           | 4.8     |
| rosmarinic acid        | \(y = 7228.7x - 0.086\)    | 0.9999                           | 0.010-20.0                        | 350            | 0.022          | 0.074          | 89.1           | 2.8     |
| trans-cinnamic acid    | \(y = 42076.6x + 3.274\)   | 0.9999                           | 0.010-24.0                        | 350            | 0.003          | 0.009          | 123.0          | 3.7     |
| quercetin              | \(y = 10036.0x + 0.293\)   | 0.9997                           | 0.050-20.0                        | 330            | 0.039          | 0.131          | 101.0          | 4.2     |
| naringenin             | \(y = 422.3x + 1.350\)     | 0.9995                           | 0.009-32.0                        | 330            | 0.057          | 0.189          | 89.5           | 2.3     |
| luteolin               | \(y = 22143.8x - 0.159\)   | 1.0000                           | 0.003-7.2                         | 350            | 0.028          | 0.087          | 90.4           | 5.1     |
| kaempferol             | \(y = 16644.4x - 0.173\)   | 0.9999                           | 0.010-27.0                        | 350            | 0.033          | 0.111          | 101.0          | 3.2     |
| apigenin               | \(y = 16431.9x - 0.095\)   | 1.0000                           | 0.008-16.0                        | 350            | 0.043          | 0.145          | 119.0          | 2.4     |
| aloe-emodin            | \(y = 14400.0x - 2.982\)   | 0.9996                           | 0.030-13.5                        | 280            | 0.029          | 0.098          | 86.2           | 2.1     |
the cleavage of glycoside bonds, with the exception of caffeic acid, which was probably partially degraded under the experimental conditions of the hydrolysis. Species of the genus *Mentha* have been reported to contain a range of phenolic components, including cinnamic acids, aglycon, glycoside or acylated flavonoids [16-18]. Eriocitrin, luteolin-7-O-glucoside, and rosmarinic acid were identified as the major components [19].

In crude ethanolic extract of buckthorn, the following compounds were identified and quantified: gallic acid, protocatechuic acid, caffeic acid, vanillic acid, chlorogenic acid, syringic acid and aloe-emodin (Table 2). Ferulic acid, myricetin, quercetin, naringenin, luteolin and apigenin were also determined after hydrolysis. As a result of hydrolysis, the content of all investigated compounds has increased, with the exception of gallic acid which showed a slight decrease in concentration, and vanillic acid, which could not be detected after hydrolysis (Table 2). From the literature data, it is known that buckthorn contains up to 8% anthraquinone glycosides of which glucofrangulins A and B, derived from the aglycone frangula-emodin, are the major compounds. Also, it is reported that buckthorn contains dianthrones, flavonoids and tannins [20-23].

In crude ethanolic extract of birch, the following compounds were identified and quantified: gallic acid, protocatechuic acid, caffeic acid, vanillic acid, chlorogenic acid, syringic acid and aloe-emodin (Table 2). Ferulic acid, myricetin, quercetin, naringenin, luteolin and apigenin were also determined after hydrolysis. As a result of hydrolysis, the content of all investigated compounds has increased, with the exception of gallic acid which showed a slight decrease in concentration, and vanillic acid, which could not be detected after hydrolysis (Table 2). From the literature data, it is known that buckthorn contains up to 8% anthraquinone glycosides of which glucofrangulins A and B, derived from the aglycone frangula-emodin, are the major compounds. Also, it is reported that buckthorn contains dianthrones, flavonoids and tannins [20-23].

In crude ethanolic extract of birch, the following compounds were identified and quantified: gallic acid,
protocatechuic acid, caffeic acid, chlorogenic acid, ferulic acid, rutin and apigenin (Table 2). Additionally, after hydrolysis, myricetin, quercetin and kaempferol were also determined. Hydrolysis caused an increase in content of gallic acid, protocatechuic acid and apigenin but a decrease in content of caffeic acid and chlorogenic acid. Significant increase in gallic acid content after hydrolysis can be explained by hydrolysis of galotannins. According to the literature data, the presence of the following compounds was determined by analysis of the birch ethanolic extracts: quercetin-3-rutinoside (rutin), quercetin-3-galactoside (hyperin), quercetin-3-glucuronide, quercetin-3-arabinopyranoside, quercetin-3-arabinofuranoside, quercetin-3-rhamnosoide and myricetin-3-galactoside [24]. Presence of myricetin-3-digalactoside, methylated flavones (acacetin and apigenin-7,4'-dimethyl ether), as well as different phenolic acids, is known in the literature [25]. Beside chlorogenic acid, neo chlorogenic acid, gallic acid, (+) catechine and glycosides of quercetin and myricetin, a presence of various other low-molecular phenolics have been determined in the birch extract [13].

HPLC analysis of phenolic compounds in the ethanolic extract of caraway has shown the presence of protocatechuic acid, caffeic acid, chlorogenic acid, (+) catechin and glycosides of quercetin and myricetin, a presence of various other low-molecular phenolics have been determined in the birch extract [13].

|                | Mentha piperitae folium | Frangulae cortex | Betulae folium | Carvi fructus | Petroselini fructus |
|----------------|-------------------------|-----------------|---------------|---------------|-------------------|
| gallic acid    | -                       | 0.20 ± 0.01     | 0.18 ± 0.02   | -             | 0.25 ± 0.01       |
| protocatechuic acid | -                     | 0.51 ± 0.02     | 0.28 ± 0.02   | 0.11 ± 0.01   | 0.11 ± 0.01       |
| caffeic acid   | 1.86 ± 0.03             | 1.14 ± 0.04     | 0.63 ± 0.04   | 2.10 ± 0.46   | 0.28 ± 0.03       |
| vanillic acid  | -                       | 1.53 ± 0.01     | -             | -             | -                 |
| chlorogenic acid | 0.73 ± 0.02            | 0.33 ± 0.01     | 0.39 ± 0.01   | 0.15 ± 0.01   | -                 |
| syringic acid  | -                       | 1.17 ± 0.02     | -             | -             | -                 |
| ferulic acid   | -                       | -               | 0.25 ± 0.03   | -             | -                 |
| rutin          | 2.18 ± 0.50             | -               | 1.24 ± 0.04   | 1.21 ± 0.11   | -                 |
| myricetin      | -                       | -               | -             | -             | -                 |
| rosmarinic acid| 12.4 ± 0.92             | -               | 0.16 ± 0.03   | 0.09 ± 0.00   | -                 |
| trans-cinnamic acid | 0.84 ± 0.02           | -               | -             | -             | -                 |
| quercetin      | 1.46 ± 0.23             | 3.58 ± 0.18     | -             | 0.55 ± 0.01   | -                 |
| naringenin     | 1.46 ± 0.23             | -               | -             | 0.36 ± 0.01   | -                 |
| luteolin       | 5.37 ± 0.38             | 17.5 ± 0.97     | 6.02 ± 0.32   | -             | -                 |
| kaempferol     | 14.9 ± 0.95             | 16.8 ± 0.02     | 7.43 ± 0.35   | 4.36 ± 0.03   | 0.18 ± 0.01       |
| apigenin       | 3.56 ± 0.26             | 1.97 ± 0.08     | 0.67 ± 0.06   | 0.52 ± 0.02   | 0.05 ± 0.01       |
| aloe-emodin    | 1.23 ± 0.01             | -               | -             | -             | -                 |

Table 2. Content of plant phenolics in crude extracts, expressed as mg g⁻¹ extract.

|                | Mentha piperitae folium | Frangulae cortex | Betulae folium | Carvi fructus | Petroselini fructus |
|----------------|-------------------------|-----------------|---------------|---------------|-------------------|
| gallic acid    | -                       | 0.16 ± 0.02     | 18.2 ± 0.39   | -             | 11.6 ± 0.45       |
| protocatechuic acid | -                     | 0.85 ± 0.01     | 0.84 ± 0.11   | -             | 0.47 ± 0.02       |
| caffeic acid   | 0.91 ± 0.05             | 2.26 ± 0.01     | 0.58 ± 0.03   | 1.54 ± 0.26   | 2.26 ± 0.13       |
| vanillic acid  | -                       | -               | -             | -             | -                 |
| chlorogenic acid | 1.66 ± 0.29            | 0.35 ± 0.02     | 0.29 ± 0.03   | 1.32 ± 0.03   | -                 |
| syringic acid  | -                       | 1.47 ± 0.10     | -             | -             | -                 |
| ferulic acid   | -                       | 0.89 ± 0.02     | 0.66 ± 0.02   | 0.52 ± 0.02   | -                 |
| rutin          | 5.37 ± 0.38             | 0.72 ± 0.01     | 6.02 ± 0.32   | -             | -                 |
| myricetin      | 17.5 ± 0.97             | -               | -             | 0.20 ± 0.09   | -                 |
| rosmarinic acid| 14.9 ± 0.95             | 1.68 ± 0.02     | 7.43 ± 0.35   | 4.36 ± 0.03   | 0.16 ± 0.01       |
| quercetin      | 3.56 ± 0.22             | 5.00 ± 0.21     | -             | -             | -                 |
| naringenin     | 13.6 ± 0.28             | 2.32 ± 0.12     | -             | -             | -                 |
| luteolin       | 0.64 ± 0.03             | 6.33 ± 0.21     | -             | -             | 2.99 ± 0.03       |
| kaempferol     | 3.56 ± 0.26             | 1.97 ± 0.08     | 0.67 ± 0.06   | 0.52 ± 0.02   | -                 |
| apigenin       | 3.48 ± 0.24             | -               | -             | -             | -                 |

Table 3. Content of plant phenolics in crude extract after hydrolysis, expressed as mg g⁻¹ extract.
caraway extracts has also been reported [27].

Quantitative analysis of phenolic compounds in the ethanolic extract of parsley has shown the presence of gallic acid, protocatechuic acid, caffeic acid, trans-cinnamic acid, luteolin, kaempferol, and apigenin. Presence of quercetin was also determined after hydrolysis, as well as increase in concentration of all other components, especially apigenin. Increase in gallic acid content after hydrolysis can be explained by hydrolysis of galotannins, as in the case of birch extracts. Sakakibara et al. [8] have reported that the main phenolic components of parsley are apigenin and glycosides of kaempferol, which is in accordance with our results. Shan et al. [28] have stated that apigenin, caffeic acid, p-coumaric acid and ferulic acid are main phenolic components of parsley but they have not been able to detect the presence of kaempferol.

The amount of total phenolics as well as the composition of the extract is highly dependent on extraction method and the type and polarity of extraction solvent [29], which could explain the differences between obtained results and literature data in some cases. Also, it is known that plant phenolic content and composition is highly affected by growing conditions and differences in the plant genotypes [19].

### 3.3 HPLC versus Folin-Ciocalteu method

Determination of total phenolic content is of particular importance for the estimation of antioxidant activity.

The most widely used Folin–Ciocalteu method is recognized as nonspecific and differentially sensitive towards different phenolic and flavonoid compounds. Also, in plant extracts, other interfering compounds, such as sugars and ascorbic acid, would contribute to the total phenolic content [4]. They would not, however, be expected to affect HPLC determined total phenols. In order to overcome the inability to quantify all the phenolic compounds present in the samples, caused by a lack of external standards, the total phenolic content of the crude ethanolic extracts was calculated as the sum of all integrated areas at 280 nm, and expressed as gallic acid equivalents, and also as the sum of all integrated areas at 350 nm, expressed as rutin equivalents. These results were compared with the total phenolic content of plant extracts determined by Folin–Ciocalteu method, and expressed as gallic acid equivalents, and with the sum of all quantified compounds after the hydrolysis (Table 4).

Although the correlation between the series of data was not significant ($p<0.05$), the difference between the results of total phenolic content obtained spectrophotometrically and HPLC results was not high in the case of parsley (Petroselini fructus), buckthorn (Frangulae cortex) and mint (Mentha piperitae folium) extract. Comparing the values of grape seed extracts in the same way, Mandić et al. [30] established highly significant correlation between the series of data, but all HPLC values were 6 times lower than spectrophotometric results.

The difference between the sum of all quantified compounds after the hydrolysis and the results of total phenolic content obtained by other approaches (Table 4) for Mentha piperitae folium can be explained by the fact that eriodictyol was not quantified due to a lack of external standards. The same explanation stands for Frangulae cortex because of the lack of external standards of emodin and its glycosides.

As the other three approaches gave similar results, the result of total phenolic content obtained by HPLC and expressed as gallic acid equivalents seems to be overestimated for the Carvi fructus sample. Kaempferol and quercetin glycosides have been reported to be the most prevalent phenolic compounds in caraway [26]. Since kaempferol and quercetin were quantified in investigated samples, the difference between the sum of all quantified compounds and total phenolic content obtained by HPLC and expressed in rutin equivalents was not high. On the other hand, the total phenolic

**Table 4.** Total phenolic content of crude plant extracts: determined by Folin–Ciocalteu method; calculated as the sum of all integrated areas at 280 nm and expressed as gallic acid equivalents; calculated as the sum of all integrated areas at 350 nm expressed as rutin equivalents; calculated as the sum of all quantified compounds after the hydrolysis.

| Sample                  | Total phenolic content by Folin-Ciocalteu method (%) | Total phenolic content by HPLC method as gallic acid equivalents (%) | Total phenolics by HPLC method as rutin equivalents (%) | The sum of all quantified compounds by HPLC (%) |
|-------------------------|-----------------------------------------------------|---------------------------------------------------------------------|------------------------------------------------------|-----------------------------------------------|
| Mentha piperitae folium | 18.4 ± 0.01                                         | 19.5 ± 0.25                                                         | 15.6 ± 0.31                                          | 6.11 ± 0.12                                   |
| Frangulae cortex        | 16.6 ± 0.08                                         | 18.0 ± 0.33                                                         | 9.01 ± 0.51                                          | 2.12 ± 0.09                                   |
| Betulae folium          | 13.9 ± 0.36                                         | 6.44 ± 0.08                                                         | 4.43 ± 0.32                                          | 2.79 ± 0.10                                   |
| Carvi fructus           | 2.89 ± 0.02                                         | 10.1 ± 0.15                                                         | 2.19 ± 0.26                                          | 1.42 ± 0.09                                   |
| Petroselini fructus     | 7.13 ± 0.86                                         | 5.43 ± 0.10                                                         | 5.77 ± 0.41                                          | 2.39 ± 0.10                                   |
content obtained spectrophotometrically for *Betulae folium* and *Petroselini fructus* was higher than that obtained by HPLC approaches.

The high correlation between the HPLC and Folin–Cioacalteu methods could be important and useful in the estimation of phenolics [31]. The HPLC data can be used as total chromatographic index (TCI) for the quantification of plant phenolics, making it a good tool towards total phenolic index (TPI) [31]. Escarpa and Gonzalez [32] published a paper, which indicated that such an approach is viable. TPI has the advantage over TPC (total phenolic content determined by the Folin–Cioacalteu method) since it gives more specific information about individual compounds or groups. Variation between the two methods was significantly lower in the study of Tsao and Yang [31], who found TPCI/TPI ratios between 1.00 and 1.30, than in that reported by Escarpa and Gonzalez [32] for all the investigated samples.

### 4. Conclusions

The new method of Rapid Resolution HPLC separation, using 1.8 µm particle size of stationary phase instead of the usual 5 µm columns, is proven to be efficient, precise, accurate, sensitive and time saving, and enabled determination of five classes of phenolic compounds present in the ethanolic plant extracts in a single run, within 22 min at ambient temperature.

The HPLC/DAD method was successfully applied to analyse the phenolic components in crude ethanolic extracts of parsley (*Petroselini fructus*), buckthorn (*Frangulae cortex*), mint (*Mentha piperitae folium*), caraway (*Carvi fructus*) and birch (*Betulae folium*) before and after hydrolysis.

Although many good HPLC methods for identification and quantification of phenolic compounds exist, total phenolic content estimation still presents a challenge.

Referring to the obtained results of the comparison between the Folin–Cioacalteu method and HPLC approach for total phenolic content estimation, the HPLC data can be used for the quantification of plant phenolics, making it a good tool towards total phenolic index, which has the advantage over total phenolic content determined by the Folin–Cioacalteu method in that it gives more specific information about individual compounds or groups at the same time.

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