POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2003, Vol. 12/53, SI 2, pp. 72-76

QUANTITATIVE AND QUALITATIVE DETERMINATION OF FLAVONOIDS AND PHENOLIC ACID DERIVATIVES FROM PERICARP OF HOT PEPPER FRUIT CV. BRONOWICKA OSTRA

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Key words: pepper; phenolics, flavonoids, phenolic acids

The derivatives of phenolic acids – ferulic and sinapic, as well as flavonoids – quercetin, luteolin and apigenin, were determined in pericarp of red pepper fruit cv. Bronowicka Ostra. Nine compounds: *trans*-*p*-feruloyl-β-D-glucopyranoside, *trans*-p-ferulylalcohol-4-*O*-(6-(2-methyl-3-hydroxypropionyl) glucopyranoside, *trans*-*p*-sinapoyl-β-D-glucopyranoside, quercetin 3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside, quercetin 3-*O*-α-L-rhamnopyranoside, luteolin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside, lutoeolin 7-*O*-[2-(β-D-apiofuranosyl)-β-D-glucopyranoside], luteolin-7-*O*-(2-apiofuranosyl--4-glucopyranosyl-6-malonyl) glucopyranoside, apigenin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside were elucidated by ^{13}C and ^{1}H NMR, MS and HPLC methods. The quantification of these compounds in pepper fruit was determined by HPLC using the isolated compounds as the standards. The obtained results showed that the greatest amounts in pericarp of hot pepper fruit var. Bronowicka Ostra were the sinapic acid glucoside and then ferulic acid glucoside, luteolin apisylglucoside and quercetin rhamnoside. The lowest level was found for apigenin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside.

INTRODUCTION

Phenolic compounds are natural products widely distributed in plants and currently consumed in large amounts in daily diet. Many nutritional studies indicated that these compounds had a good influence on human health [Bartnikowska, 1995; Cook & Samman, 1996; Peterson & Dwyer, 1998; Czeczot, 2000; Harborne & Williams, 2000]. Phenolic acids, flavonoids and anthocyanins and their derivatives are known as good antioxidants. The most advantageous effect of phenolic compounds in plants depend on ascorbic acid protection [Okuda, 1993]. In *in vitro* models they inhibit LDL oxidation. This effect is due to their radical scavenging and metal ions chelating capabilities. Additionally polyphenols are found as being beneficial against cancer and other civilization diseases. For this reason, knowledge about phenolic compounds in popular fruits and vegetables is necessary. In the present work, fruits of hot pepper (*Capsicum annuum* L.) var. Bronowicka Ostra have been studied with regard to the content of flavonoids and other phenolics. Many studies have been carried out on accumulation of vitamins and carotenoids [Palevitch & Craker, 1995; Daood *et al*., 1996; Perucka, 1996; Hornero--Mendez *et al*., 2000; Lee & Kader, 2000], but information on flavonoids and phenolic acids in pepper fruits is scarce [Sukrasno & Yeoman, 1993; Lee *et al*., 1995; Perucka & Materska, 2003]. Thus, the aim of this study was to isolate and determine flavonoid and phenolic acid derivatives occurring in pericarp of pepper fruit.

MATERIALS AND METHODS

Fruits of hot pepper variety Bronowicka Ostra were taken for the study at the full ripeness stages. Freeze-dried material (equivalent to 1 kg fresh fruits) was homogenized with 80% EtOH according to Lee *et al*. [1995]. The phenolic compounds were isolated from pepper extract, as described in detail by Materska *et al*. [2003].

The HPLC analysis was made on Waters chromatograph with a PDA 996 detector, a 616 pump and the Millenium programme. Separations were performed on an RP-18 (Eurospher 80, Säulentechnik 250 x 4.6 mm, 5 μm) column and at 1 mL/min solvent delivery. The linear gradient of 0-40% acetonitrile (CH_3CN) in 1% H_3PO_4 was used as a solvent system. The preliminary identification of phenolic compounds was conducted on the basis of UV spectra recorded on PAD detector during chromatographic analysis.

The spectra ^{1}H NMR and ^{13}C NMR were recorded in CD_3OD on Bruker DRX-600 spectrometer working at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR.

Mass spectra ESI-TOF were recorded on Mariner spectrometer (PerSeptive Biosystems).

The standard solutions of pure phenolic compounds isolated from extract of pepper fruit at concentration ranging from 3 $x10^{-4}$ mol/L to 8 $x\ 10^{-4}$ mol/L for each compound were prepared. The concentration of analysed compounds in pericarp of red pepper fruits was determined by HPLC using the external standard curve method.

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TABLE 1. Retention data, spectral characteristic from photodiode array detection and ESI-TOF MS spectral data for isolated phenolic compounds.

	Compound number								
	1	2	3	4*	5	6	7	8	9*
Retention time (min)	13.25	14.52	17.10	17.10	18.20	22.50	26.28	30.28	32.41
Molecular mass	356	386	598	428	580	564	580	448	828
Abs. maximum (nm)	219 238 328	238 328	210 257 347	210 257 347	215 271 352	219 271 333	205 257 347	210 257 347	205 257 347
Positive ion (m/z):									
$[M+H]^+$	357	387	599	429	581	565	581	446	829
$[M+Na]^+$	379	409		451	603			471	
$[M+K]^+$			634						
$[M+NH_4]^+$				446					
Negative ion:									
$[M-H]^-$	355	385	597	427	579	563	579	447	827
$[M+Cl]^-$					615			483	

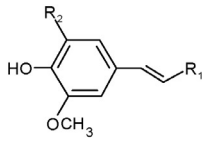
* Data cited from Materska *et al*. [2003].

RESULTS AND DISCUSSION

Introductory analysis of 40% MeOH fraction isolated from hot pepper fruits showed its fairly complex composition. After preparative separation and purifying particular fractions nine pure compounds were obtained.

The spectrum of one of the main compounds (**1)** recorded with a PDA detector was characterized by three absorption maxima; the main one at λ = 328 nm and two smaller ones at 219 and 238 nm. On the basis of mass spectrometry analysis the formula of compound **1** was determined as $C_{16}H_{20}O_9$. A ^{13}C NMR analysis confirmed that formula, suggesting the presence of ten carbon atoms in the non-sugar part and six ones in the sugar unit (Table 2). In ^1H-NMR spectrum, six proton signals in the range of 6.43-7.76 ppm corresponded to an aglycone part and the remaining signals within the range of 3.21-5.60 ppm corresponded to glucose. On this basis, it was found that compound **1** is a *trans*-p-feruloyl-β-D-glucopyranoside (Figure 1).

The second main component of the pepper fruit was *trans*--p-sinapoyl-β-D-glucopyranoside (Figure 1). The UV spectrum of this compound was similar to the spectrum of compound **1**, with the main absorption maximum at 328 nm; the second maximum occurred at 238 nm. On the basis of the ESI-TOF MS analysis the formula of compound **2** was determined as $C_{17}O_{10}H_{22}$ (Table 1). The ^{13}C NMR analysis confirmed the presence of 17 carbon atoms in the molecule, 11 of which belonged to the non-sugar part (Table 2). In the case of ^1H NMR analysis the differences in signals as compared to compound **1** were observed only in the non-sugar part where a lack of proton resonance signals in position 3 and appearance of an additional singlet signal for group O-Me proves that hydrogen was replaced in this position by a metoxyl substituent.



Peak No	Phenolic acid derivatives	R_1	R_2
1	*trans-p*-feruloyl-β-D-glucopyranoside	-CO-Glc	-H
2	*trans-p*-sinapoyl-β-D-glucopyranoside	-CO-Glc	$-OCH_3$

FIGURE 1. Phenolic acid derivatives identified in the extracts from pericarp of red pepper fruits *Capsicum annuum* L.

In the case of compounds **3** and **4**, the UV spectrum was recorded for their mixture. In the spectrum, the presence of three maxima of absorption was found, at 210, 257 and 347 nm. The formula of compound **3** determined on the basis of mass spectra was $C_{26}O_{16}H_{30}$. A ^{13}C NMR analysis confirmed the number of carbon atoms in the molecule (Table 1), and a ^1H NMR analysis showed that part of signals situated within the range of 7.41-6.54 ppm corresponds to an aglycone unit, whereas signals situated in the range of 5.41-0.97 ppm to protons in the sugar part. On the basis of the literature data [Markham, 1982], it was determined that the aglycone occurring in compound **3** is quercetin and the remaining 12 signals in spectrum ^{13}C NMR came from the sugar part. A comparison of chemical shifts in spectrum ^{13}C NMR of quercetin with the standard spectrum of this compound [Markham, 1982] showed that sugar substituents were situated in positions C-3 and C-7 (Table 2). The sugars were rhamnose and glucose, so compound **3** is quercetin 3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside (Figure 2).

The data on structure elucidation of *trans-p*-ferulylalcohol-4-*O*-(6-(2-methyl-3-hydroxypropionyl) glucopyranoside (coniferin 6'-*O*-2-methyl-3-hydroxypropionyl) – compound **4** (Figure 3), were presented in our earlier paper [Materska *et al*., 2003].

The next compound with the retention time of 20 min was luteolin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside (Figure 2). The UV spectrum recorded on PAD detector had three absorption maxima: 215, 271 and 352 nm. On the basis of the data obtained from mass spectrometry analysis the empirical formula of compound **5** was determined as $C_{26}O_{15}H_{28}$. A ^{13}C NMR analysis confirmed the number of carbon atoms in a mol-

TABLE 2.The ^{13}C NMR data of the isolated compounds.

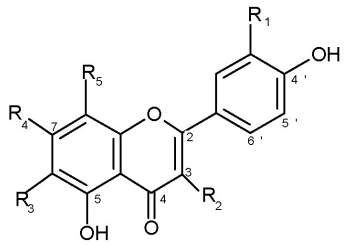
δ (ppm)										
C	**1**	**2**	**4***	C	**3**	**5**	**6**	**7**	**8**	**9***
Phenolic acids				Aglyconees						
1	127.4	126.2	133.8	2	159.9	165.3	165.3	166.8	159.8	166.8
2	124.3	106.8	120.5	3	135.0	103.6	103.6	104.1	135.1	104.1
3	116.4	149.1	117.3	4	178.9	183.5	183.6	184.1	178.7	184.1
4	150.5	139.6	147.0	5	162.0	161.0	160.6	162.9	162.2	162.9
5	149.5	149.1	150.7	6	100.0	100.0	109.4	101.3	101.3	101.3
6	111.5	106.8	111.0	7	164.5	163.2	163.0	164.5	166.5	164.5
α	115.3	115.0	131.0	8	95.7	105.3	105.6	96.1	97.2	96.1
β	147.0	148.3	128.7	9	157.6	155.5	155.5	158.9	158.0	158.9
C=O	168.5	167.5	63.2	10	107.6	104.6	104.5	107.3	106.8	107.3
OMe	56.8	56.8	56.2	1’	120.9	122.8	122.5	123.1	120.9	123.1
				2’	116.6	115.0	130.2	114.3	116.6	114.3
				3’	141.1	147.0	117.2	147.1	146.1	147.1
				4’	143.9	151.2	162.5	151.2	143.9	151.2
				5’	115.7	117.4	117.2	116.8	115.7	116.8
				6’	122.6	121.1	130.2	120.5	122.6	120.5
				Sugars						
1’	95.9	95.9	101.8	1”	102.7	75.7	75.7	100.8	102.7	101.0
2’	75.0	75.0	74.2	2”	71.4	72.9	72.9	78.5	71.4	78.2
3’	77.6	77.6	77.4	3”	71.4	79.9	79.9	78.3	71.4	76.8
4’	71.0	71.0	70.7	4”	72.6	69.5	69.5	71.4	72.4	80.4
5’	78.5	78.5	74.9	5”	70.7	82.3	82.3	78.1	70.5	77.8
6’	62.3	62.3	64.4	6”	18.0	61.3	61.3	62.5	17.6	64.6
				1”’	100.0	76.0	76.0	110.7		110.2
				2”’	74.2	70.0	70.0	78.1		78.1
				3”’	77.9	76.5	76.5	80.8		80.6
				4”’	71.4	70.1	70.1	75.47		75.5
				5”’	77.3	71.7	71.7	65.7		65.9
				6”’	61.9					
				1””						103.5
				2””						74.9
				3””						77.8
				4””						69.8
				5””						77.8
				6””						62.0
				Other						
1”			176.1	1^V						168.7
2”			43.2	2^V						42.1
3”			64.4	3^V						170.3
4”			12.8							

* Data cited from Materska *et al*. [2003].

ecule; it was found that 15 carbon atoms belonged to the non-sugar part (Table 1). A comparison of ^{13}C NMR and ^{1}H NMR spectra with the literature data [Markham, 1982] shows that the aglycone in the molecule was luteolin. A downfield shift at C-6 (δ=100) and C-8 (δ=105.3) of luteolin proves C-glycosidic bonds with sugar units.

Compound **6** had UV spectrum similar to compound **5** with three absorption maxima at 219, 271 and 333 nm (Table 1). On the basis of the results obtained from a mass spectrometry analysis, the compound formula was determined as $C_{26}O_{15}H_{28}$. A comparison of ^{1}H and ^{13}C NMR spectra with the spectra of compound **5** (Table 2) and with the literature data [Markham, 1982] shows that the compounds **5** and **6** differ from each other only in the kind of aglycone. In compound **5** the aglycone was luteolin and in the case of compound **6** it was apigenin. Hence, compound **6** is apigenin 6-*C*-β-D-glucopyranoside-8--*C*-α-L-arabinopyranoside known as the schaftoside (Figure 2) [Harborne & Mabry, 1982].

Compound **7** was lutoeolin 7-*O*-[2-(β-D-apiofuranosyl)--β-D-glucopyranoside] (Figure 2). The UV spectrum of this compound contained three absorption maxima at 205, 257 and 347 nm. On the basis of an ESI-TOF MS analysis (Table 1), the molecule formula was determined as $C_{26}O_{15}H_{28}$. A ^{13}C NMR analysis (Table 2) confirmed the number of carbon atoms in a molecule. It was found that 15 carbon atoms were in the non-sugar part and 11 atoms in the sugar one. In the case of ^{1}H NMR analysis, the signals situated within the range of 6.51-7.45 ppm belonged to the aglycone part and the signals within the 3.57-5.49 ppm came from the sugar unit. A comparison of ^{13}C NMR spectra of compound **7** with **5** showed occurrence of luteolin. The attachment of the sugar moiety to C-7 of the aglycone was deduced from the chemical



Peak No	Compound	R_1	R_2	R_3	R_4	R_5
3	quercetin 3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside	-OH	-O-Rha	-H	-O-Glc	-H
5	luteolin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside	-OH	-H	-C-Glc	-OH	-C-Ara
6	apigenin 6-*C*-β-D-glucopyranoside-8-*C*-α-L-arabinopyranoside	-H	-H	-C-Glc	-OH	-C-Ara
7	lutoeolin 7-*O*-[2-(β-D-apiofuranosyl)-β-D-glucopyranoside]	-OH	-H	-H	-O-Glc-Api	-H
8	quercetin 3-*O*-α-L-rhamnopyranoside	-OH	-O-Rha	-H	-OH	-H

FIGURE 2. Flavonoid derivatives identified in the extracts from pericarp of red pepper fruits *Capsicum annuum* L.

shifts in that position. A comparison of ^{13}C NMR spectra of the sugar part confirmed the presence of glucose and apiose, the downfield shift at C-2'' (δ=78.5) of the glucose proves occurrence of the 1→2 bond between the sugar units (Table 2).

The next isolated compound was quercetin 3-*O*-α-L-rhamnopyranoside (Figure 2). The absorption maxima in the UV spectrum occurred at 210, 257 and 347 nm. On the basis of the molecular ions obtained in an MS analysis, the empirical formula of compound **8** was determined as $C_{21}O_{11}H_{20}$. The ^{13}C NMR analysis (Table 2) confirmed the number of carbon atoms in a molecule. In the ^{1}H NMR spectra, it was found that the signals situated within the range of 6.51-7.45 belonged to aglycone part, and the signals situated within the range of 3.56-5.49 – to the sugar unit. A comparison of the mass spectra and ^{13}C NMR and ^{1}H NMR of compound **8** with the literature data [Markham, 1982; Ossipov *et al*., 1995; Ryan *et al*., 1999; Steeves *et al*., 2001] suggests that the aglycone in the molecule was quercetin, and the sugar part was constituted by rhamnose.

The UV spectrum of compound **9** had three absorption maxima, at 205, 257 and 347 nm. A mass spectrometry analysis showed the empirical formula of compound **9** is $C_{35}O_{23}H_{46}$. The ^{13}C NMR spectrum of the compound revealed the presence of 17 carbon atoms belonging to sugar substituents. Compound **9** is luteolin 7-*O*-[2-(β-D-apiofuranosyl)-4-(β-D-glucopyranosyl)-6--malonyl]-β-D-glucopyranoside [Materska *et al*., 2003].

The results of the determination of the individual amounts of isolated compounds showed that the contents of phenolic acid derivatives in pericarp of red pepper fruits of Bronowicka Ostra were from 40 to 57 mg/kg f.w. and flavonoids from 2.5 to 34 mg/kg f.w. (Figure 4). The main flavonoid derivatives were lutoeolin 7-*O*-[2-(β-D-apiofuranosyl)-β-D-glucopyranoside] about 35 mg/kg f.w. and quercetin 3-*O*-α-L-rhamnopyranoside 20 mg/kg f.w. The lowest level, 2.5 mg/kg f.w.,

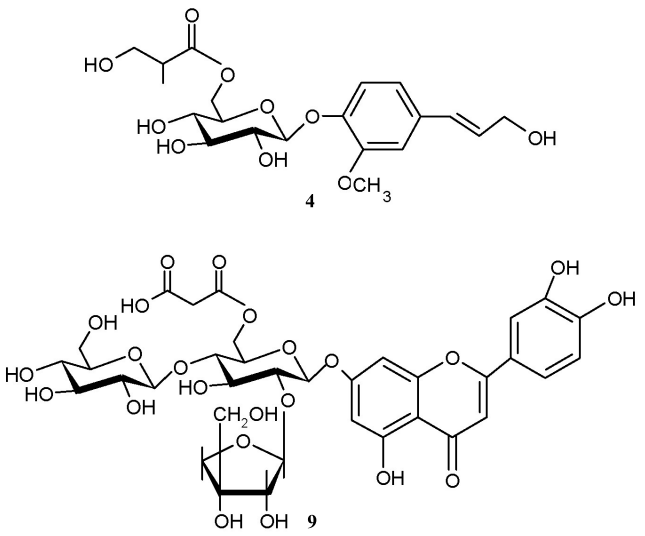


FIGURE 3. Chemical formula of the new compounds identified in red pericarp *Capsicum annuum* L. 4: *trans*-p-Ferulylalcohol-4-*O*-(6--(2-methyl-3-hydroxypropionyl) glucopyranoside; 9: Luteolin-7-*O*--(2-apiofuranosyl-4-glucopyranosyl-6-malonyl) glucopyranoside.

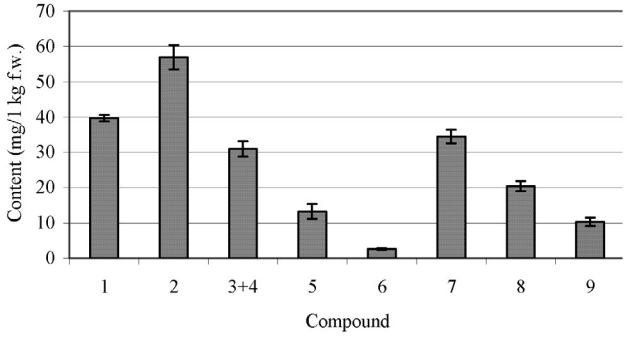


FIGURE 4. The content of isolated phenolic compounds in pericarp of hot pepper fruit var. Bronowicka Ostra (SD, n=3). In compounds 3+4, the standard solution was prepared from the mixture of these compounds.

was found for apigenin 6-*C*-β-D-glucopyranoside-8-*C*-α-L--arabinopyranoside. For luteolin 6-*C*-β-D-glucopyranoside--8-*C*-α-L-arabinopyranoside the content was nearly the same (13.3 mg/kg f.w.) as for luteolin-7-*O*-(2-apiofuranosyl-4-glucopyranosyl-6-malonyl) glucopyranoside (10.3 mg/kg f.w.). Mixture of quercetin 3-*O*-α-L-rhamnopyranoside-7-*O*--β-D-glucopyranoside and *trans*-p-ferulylalcohol-4-*O*-(6-(2--methyl-3-hydroxypropionyl) glucopyranoside amounted to 31 mg/kg of pericarp of fresh weight fruits of hot pepper var. Bronowicka Ostra.

The amounts of these compounds were assayed except for lutoeolin 7-*O*-[2-(β-D-apiofuranosyl)-β-D-glucopyranoside] and quercetin 3-*O*-α-L-rhamnopyranoside for the first time in pepper fruits. The compound **7** and **8** were in the near the same amounts as mentioned by Sukrasno and Yeoman [1993]. The levels of 7-*O*-[2-(β-D-apiofuranosyl)-β-D-glucopyranoside] luteolin as 7-*O*-glucosylluteolin and 3-*O*-rhamnosylquercetin were determined by Sukrasno and Yeoman [1993], but their results were higher than the ones obtained in this study. The differences may result from the methods of isolation and quantification of those compounds. These researches estimated the amount of conjugated phenolics using aglycones as standards.

CONCLUSIONS

The nine derivatives of phenolics were isolated and quantified in pericarp of red pepper fruit var. Bronowicka Ostra. One of them, luteolin-7-*O*-(2-apiofuranosyl-4-glucopyranosyl-6-malonyl) glucopyranoside was determined for the first time in the plant kingdom. It was found that the dominating phenolic compounds were feruloyl and sinapoyl glucosides as well as luteolin apiosylglucoside and quercetin rhamnoside. The contents of the remaining compounds were lower and constituted less than 38% of the determined derivatives of phenolic acids and flavonoids.

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