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An improved HPLC-DAD method for simultaneously measuring phenolics in the leaves of *Tilia platyphyllos* and *Ailanthus altissima*

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ABSTRACT: Phenolic compounds are one of the most important groups of secondary metabolites in plants, with various physiological functions. Their diverse chemical structure and susceptibility to auto-oxidation, and their ability to act as both antioxidants and prooxidants in the presence of metal ions, are some of the main reasons why it is difficult to measure phenolic groups in plant tissues accurately. We present an optimized extraction and hydrolysis procedure which preserves the original chemical structure of phenolics. The presented HPLC method was improved to enable the simultaneous separation and quantification of 39 compounds from different phenolic subclasses (benzoic acids, hydroxycinnamic acids, flavones, flavonols, flavanones, flavanols, isoflavones, anthocyanidins; aglycones and glycosides). Recovery after extraction and complete hydrolysis of glycosides was more than 95% and 84%, respectively. The method was applied to the analysis and comparison of phenolic profiles in the leaves of two species, *Ailanthus altissima* (Mill.) Swingle and *Tilia platyphyllos* Scop. from an urban forest park and busy traffic area. The presence of cyanidin glycosides in *A. altissima* leaves was reported for the first time. Results indicated higher accumulation of phenolics, with two hydroxyl groups in the *ortho-* position, than flavonoids, with a monohydroxy substitution in the leaves of both species from a busy traffic area.

Key words: HPLC method development; anthocyanins; flavonoid glycosides; phenolic acids, phenolic profiles; urban environment.

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INTRODUCTION

Phenolic compounds are a widespread class of secondary metabolites with numerous structural and protective physiological functions in plants. Phenolics can act as sunscreens in the leaf epidermis; they are involved in signaling pathways, protection against herbivores and pathogens, scavenging of reactive oxygen species, cell wall strengthening (lignin) and protein tanning (Dixon & Paiva 1995; Cerovic *et al.* 2002; Gould & Lister 2005; Pollastri & Tattini 2011). Almost 20% of the carbon fixed in photosynthesis is used for phenolic biosynthesis in the phenylpropanoid pathway with phenylalanine or tyrosine precursors (Ralston *et al.* 2005).

The feature common to all phenolic compounds is the presence of at least one hydroxy-substituted aromatic ring that forms a carbon skeleton building block: the C_6-C_3 phenylpropanoid unit (Pereira *et al.* 2009). Depending on the complexity of their chemical structure, the great variety of naturally-occurring phenolics can be divided into: benzoic acids (C_6-C_1), cinnamic acids (C_6-C_3), coumarins (C_6-C_3), flavonoids (C_6-C_3-C_6), proanthocyanidins [(C_6-C_3-C_6)n], stilbenes (C_6-C_2-C_6), lignans (C_6-C_3-C_3-C_6) and lignins [(C_6-C_3)n] (Antolovich *et al.* 2000; Marais *et al.* 2006). Depending on the nature of the C_3 element, flavonoids can be divided into flavones, flavonols, flavanones, flavanols, isoflavones, chalcones and anthocyanidins (Pereira *et al.* 2009).

Phenolic hydroxyl groups are good hydrogen and electron donors; therefore they can react with reactive oxygen species (ROS) and reactive nitrogen species (RNS) by forming more stable, long-lived radicals. (Rice-Evans

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et al. 1996; Pereira *et al.* 2009). This stabilization is usually achieved through delocalization, intramolecular hydrogen bonding or further oxidation in a reaction with another radical, leading to polymerization and lignin biosynthesis (Croft 1998). Together with ascorbic acid and the Class III peroxidases, phenolics - and flavonols and hydroxycinnamic acids in particular - form a highly-efficient cycle for scavenging free radicals in the vacuole and intercellular space (Takahama 2004). Phenolic compounds with two *ortho*-dihydroxyl groups or other chelating structures can bind transition metal ions (especially iron and copper), contributing to their antioxidant capacity (Rice-Evans *et al.* 1996). However, phenolics can also act as pro-oxidants by increasing metal catalytic activity after chelation, or by reducing metals (Cu^{2+} to Cu^{+}) and thereby enabling the formation of initiating radicals (Cao *et al.* 1997; Decker *et al.* 1997).

Exposure to various abiotic and biotic stresses can modify species specific phenolic profiles (Dixon & Paiva 1995; Agati & Tattini 2010). Under such unfavorable conditions, redox homeostasis in the cells is altered due to the accumulation of oxidative species, such as H_2O_2, which may initiate phenolic auto-oxidation. Establishing the levels of phenolic compounds in plant tissues is particularly difficult due to the structural diversity of phenolics, which affects their physical and chemical properties, e.g. their solubility, susceptibility to hydrolysis, (photo)oxidation and thermal degradation (Häkkinen *et al.* 1998; Antolovich *et al.* 2000). Although a large number of methodological reports have been published on the qualitative and quantitative analysis of different types of phenolic compounds using C18 reversed phase high-performance liquid chromatography (HPLC) for separation, most of the studies, however, referred to just one or a few phenolic subclasses or similar plant species (Häkkinen *et al.* 1998; Molnár-Perl & Füzfai 2005; Lee *et al.* 2008; Haghi & Hatami 2010). Very few studies covered a broad range of phenolic compounds in different plant materials and, in those that did so, the analyses took a long time to complete (60–95 min) and the separation process was inadequate (Crozier *et al.* 1997; Merken & Beecher 2000; Sakakibara *et al.* 2003; Shan *et al.* 2005). HPLC coupled with a diode array detector (HPLC–DAD) presents a cheaper and more accessible analytical tool (compared with other detectors which are widely coupled to HPLC) which can provide very good separation and identification of a wide range of phenolic compounds.

Accordingly, the objectives of the present work were to optimize extraction and hydrolysis conditions and establish an efficient and reliable HPLC–DAD method for rapidly extracting, hydrolysis and simultaneous characterization of all phenolic subclasses, both aglycones and their glycosides, for various plant species while preserving the phenolics' diverse chemical structures. The developed HPLC–DAD method was validated by analysis of leaves from four species: Swedish ivy (*Plectranthus coleoides* Benth.), pelargonium (*Pelargonium zonale* L. cv. "Ben Franklin"), the tree of heaven (*Ailanthus altissima (*Mill.) Swingle) and linden (*Tilia platyphyllos* Scop.). Furthermore, the method was applied to investigate specific phenolic profiles under urban conditions in two tree species, *A. altissima* and *T. platyphyllos*, commonly found in green spaces in Belgrade, Serbia. *A. altissima* is one of the most tolerant tree species of urban stress factors (Trifilò *et al.* 2004; Kowarik & Säumel 2007), while *T. platyphyllos* was described as sensitive to industrial and urban pollutants (Kropczynska *et al.* 2002; Fini *et al.* 2009).

MATERIAL AND METHODS

Plant material. Pelargonium (*P. zonale*) and Swedish ivy (*P. coleoides*) were grown under controlled conditions (day/night cycle, 16/8 h, 300 μmol m^{-2} s^{-1} and 25°C). Leaf samples of linden (*T. platyphyllos*) and the tree of heaven (*A. altissima*) were taken from trees in the Kosutnjak forest park (44°48′30″N, 20°27′49″E, altitude 250 m) in Belgrade, and from trees along Kneza Milosa Street (44°47′21″N, 20°27′59″E, altitude 149 m), one of the most polluted streets in Belgrade with very busy traffic, in August 2012. The concentrations of gases and particles in Kneza Milosa Street, emitted mainly by vehicle congestion, are presented in Appendix Table I.

Chemicals and reagents. Standard substances - including gallic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, benzoic acid, *p*-hydroxy benzoic acid, coniferyl alcohol, apigenin, naringenin, luteolin, (+)-catechin, (–)-epicatechin, resveratrol, myricetin, quercetin, daidzein, genistein, naringenin, and pelargonidin - were obtained from Sigma-Aldrich GmbH (Germany); *m*-coumaric acid, protocatechuic acid, ferulic acid, syringic acid, ellagic acid, quercetin-3-*O*-rutinoside (rutin), apigenin-7-*O*-glucoside came from Fluka (Switzerland); myricetin and sinapic acid were obtained from Acros Organics (Belgium); kaempferol, quercetin-3-*O*-glucoside, delphinidin, cyanidin, pelargonidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinose, naringenin-7-*O*-rutinoside were obtained from Extrasynthese (France). All chromatographic solvents were HPLC grade. For the calibration curves, stock solutions of the standards were freshly prepared within the range of 0.2 to 20 mg mL^{-1} by dissolving pure compounds in methanol containing 0.1% HCl or in methanol containing 2 mol dm^{-3} HCl. The concentration of the standard solutions was selected according to the level of the analytes expected in the samples.

Extraction and hydrolysis. Leaves of *P. zonale*, *P. coleoides, T. platyphyllos* and *A. altissima* were frozen in liquid nitrogen immediately after collection and rapidly ground with a pestle and mortar, then extracted in

methanol containing 0.1% HCl, incubated for 50 min on ice in the dark, and centrifuged for 10 min at 16,000 *g* and 4 ˚C. Supernatants (600 µL) were mixed with ddH_2O (400 µL) and chloroform (600 µL), and shaken for 45 min at 4 ˚C in the dark. The samples were then centrifuged for 5 min at 16,000 *g* and 4 ˚C to separate the liquid phases. The upper, water layer was split in two halves. The first half was used for glycoside determination. The second half was hydrolyzed in 2 mol dm^{-3} HCl during incubation at 85 ˚C for 40 min and 100 min for optimization, according to a modified version of the method described by Hertog *et al.* 1992. At the same time, the procedure was repeated without adding chloroform to obtain the best extraction conditions. Finally, all extracts were flushed with nitrogen and stored at -80 ˚C until HPLC analysis.

Linearity, quantification, limit of detection (LOD) and limit of quantification (LOQ). Calibration curves were constructed by means of a linear regression of five different concentrations of standard phenolic compounds, repeated in triplicate. The limit of detection (LOD) and quantification (LOQ) for each analyte were determined by injecting serial dilutions of the corresponding standard solution and calculated based on a signal-to-noise ratio of 3 for LOD and signal-to-noise ratio of 10 for LOQ. Quercetin, myricetin, kaempferol, rutin, pelargonidin, pelargonidin-3-*O*-glycoside, cyanidin-3-*O*-glycoside and caffeic acid (all 1 mg mL^{-1}) standards were processed in triplicate, in the same way as the plant samples, as described above.

Repeatability and recovery. Repeatability was determined based on analysis of injection of the standard solution with four repetitions. For recovery, powdered leaf samples of *P. zonale, P. coleoides, T. platyphyllos* and *A. altissima* were divided into two equal parts: one was extracted in methanol with 0.1% HCl as described above; 20 µL aliquots of quercetin, myricetin, kaempferol, pelargonidin, caffeic acid, rutin, pelargonidin-3-*O*-glycoside, cyanidin-3-*O*-glycoside (all 1 mg mL^{-1}) were added to the other half, followed by extraction in methanol with 0.1% HCl. The complete procedure was repeated in triplicate in both cases, with and without the addition of chloroform. Recovery was calculated after subtracting the average peak area of extract/hydrolysate from the average peak area of the sample with the internal standards, and the resulting peak area was compared with those obtained with the pure standard solutions.

Apparatus. Reversed-phase HPLC was performed using a Shimadzu LC-20AB Prominence liquid chromatograph (Kyoto, Japan) consisting of binary pumps LC-20 AD pumps, a CTO-20A column oven, a DGU-20A3 degasser, an SPD-M20A diode array Prominence detector and a SIL-20 AC auto-injector. Components were separated on a Luna C18 (2) 100Å (250 mm x 4.6 mm i.d., 5 µm) column (Phenomenex UK, Macclesfield) at 25 °C. The detector was set at different wavelengths, depending on the characteristic maximum absorbance of the selected phenolics: 520 nm for anthocyanins, 360 nm for flavonols and ellagic acid, 340 nm for flavones and flavanones, 320 nm for coniferyl alcohol, resveratrol, hydroxycinnamic acids and their derivatives, and 280 nm for catechins, isoflavones, benzoic acids and their derivatives. Absorption spectra were recorded for each peak. Data were plotted and peak areas calculated with a Shimadzu 'LC Solution'.

HPLC conditions. The HPLC analysis was performed according to a modified version of the method (Lee *et al.* 2008), finally optimized for a broad range of phenolics. Elution solution A was acetonitrile and elution solution B contained acetic acid/acetonitrile/phosphoric acid/water (10:5:0.1:84.9, v/v/v/v). The following elution procedure was found to achieve an acceptable separation of all compounds (Table I): 0–5 min, 100% solution B isocratic step); 5–25 min, 100–80% solution B (linear gradient); 25–35 min, 80–60% solution B (linear gradient); 35–40 min, 60–100% solution B (linear gradient) at a flow rate of 1 mL min^{-1} and constant temperature of 25 °C. The injection volumes were 10 µL and 50 µL for the standards and extracts, respectively.

Statistical analysis. Data were analyzed using the Statistica software (Version 6.0, StatSoft) for Windows. The significance of the differences between untreated and treated plants was tested with Student's *t* –test. The threshold for significance was set at $P \leq 0.05$.

Table I. HPLC eluent gradient for phenolics separation and determination.

Time / min	Solvent A / %	Solvent B / %
0	0	100
5	0	100
25	20	80
35	40	60
40	0	100

RESULTS AND DISCUSSION

Analysis of phenolic compounds. Several preliminary analyses were run to develop the most appropriate HPLC method for separating a broad range of phenolic compounds in different plant materials. The optimized composition of the mobile phase enabled a satisfactory resolution in the separation of 39 different phenolic compounds belonging to the following groups: phenolic acids, flavanols, isoflavones, flavanones, flavones, flavon-

3-ols, anthocyanidins and their glycosides (Fig. 1). The spectral characteristics and retention times for each standard solution are listed in Table II.

The analytes were identified by matching the retention times and spectral characteristics against standard solutions and details reported in the literature (Sakakibara *et al.* 2003; Giusti & Wrolstad 2001). Samples were spiked with standard solution for confirmation. In the case of glycosides for which standards were not readily available, peaks were ascertained based on the spectrum and elution order, and on data in the literature on the glycoside forms to be expected in the species analyzed (Toker *et al.* 2001; 2004; Kundu & Laskar 2010; Said *et al.* 2010) and compounds already identified in various other plant species and fruits (unpublished data).

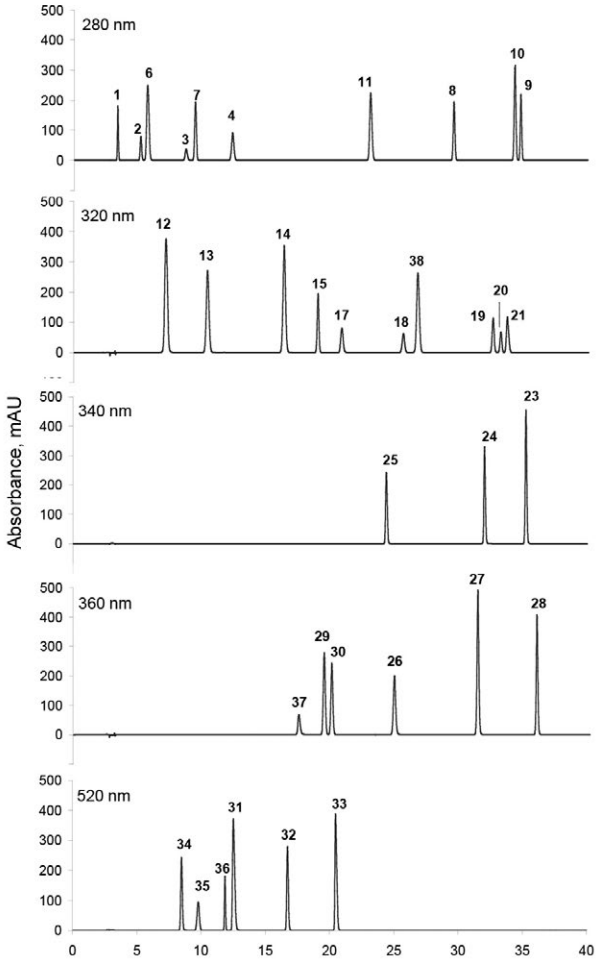

Fig. 1. HPLC–PDA chromatograms of phenolic standard mixtures detected at (A) 280 nm; (B) 320 nm; (C) 340 nm; (D) 360 nm; (E) 520 nm. Peak numbers refer to Table II.

The calibration curve was constructed for each of the standards by plotting the concentration of the standard (μmol dm^{-3}) against the peak area at the group-specific wavelengths (Table II). Each standard gave an almost linear calibration curve through the zero point. The slopes of the calibration curves and the limits of detection and quantification are shown in Table II. The lowest limit of quantification found for the reference compounds was for gallic acid (28 pmol) and the highest for epicatechin (530 pmol) (Table II).

Optimization of extraction and recovery. In our study, the best results were obtained with a rapid extraction in methanol containing 0.1% HCl under a nitrogen-rich atmosphere, followed by re-extraction with chloroform. The reproducibility of the extraction procedure was satisfactory, in the range of 5-10%. Adding chloroform improved the purification of the extracts and prevented any interference from hydrophobic compounds such as chlorophyll and fatty acids. In samples extracted with chloroform we obtained a better resolution, base line and selectivity, but the kaempferol yield decreased by 10-30% (Table III). Adding chloroform did not affect the content of extracted flavonoid glycosides, as the recovery was 100.6% for cyanidin-3-*O*-glucoside and 104.9% for quercetin-3-*O*-rutinoside. The same results were obtained in the external standard solutions (data not shown).

Hydrolysis and recovery. Phenolic acids and flavonoids are usually found in plants in the form of glycosides (Croft 1998). Sample hydrolysis is needed prior to analysis to ascertain the glycosides' structure and other characteristics (Nuutila *et al.* 2002; Antolovich *et al.* 2000). The most common method for assessing flavonoid glycosides is hydrolysis using HCl in methanol under continuous heating (Hertog *et al.* 1992; Merken & Beecher 2000). The incubation time during hydrolysis is essential in this case because the conditions resulting in the optimal breakdown of glycosides may be too harmful for some of the other phenolic compounds in the same plant material (Nuutila *et al.* 2002). The recovery of standard solutions during hydrolysis was therefore measured after 40 and 100 min of incubation at 85 ˚C in methanol containing 2 mol dm^{-3} HCl. After 40 min of hydrolysis, the recovery rate was above 90%, except for kaempferol (66–86%) and myricetin (87–94%); see Table III. After 100 min of hydrolysis, the recovery rate was 10-15% lower than after 40 min for all tested compounds. Based on these results, 40 min was chosen as the optimal hydrolysis time. These conditions were also found suitable in quantitative terms for the purpose of glycoside hydrolysis: after 40 min, 0.66 nmol of quercetin-3-*O*-rutinoside was completely hydrolyzed, while 97.6–98.6% of anthocyanins (0.30 nmol of cyanidin-3-*O*-glucoside and 0.30 nmol of pelargonidin-3-*O*-glucoside standard solutions) had been converted into their aglycone forms. The hydrolysis time would need to be longer (100 min), however, in the case of samples containing larger amounts of anthocyanins, *i.e.* more than 50 μg per g of fresh weight, such as berry fruits (unpublished data).

Table II. List of standard compounds used to create calibration curves

Subclass	Phenolic compound	No.	[a] λ / nm	[b] Rt / min	Spectral characteristics / nm	[d] slope (x10^{-4})	[e] LOD /pmol	[f] LOQ /pmol
benzoic acids	gallic acid	1	280	3.39	218, 271	0.438	8	28
	protocatechuic acid	2	280	5.17	239, 259, 294	0.343	17	56
	p-hydroxy benzoic acid	3	280	8.70	239, 255	0.150	32	106
	syringic acid	4	280	12.33	225, 274	0.503	19	62
	benzoic acid	5	280	24.61	242, 273	0.061	25	84
flavanols	(+)-catechin	6	280	5.71	241, 278	0.188	125	416
	(−)-epicatechin	7	280	9.43	240, 278	0.185	159	530
isoflavones	daidzein	8	280	29.57	262, 302	0.357	153	510
	genistein	9	280	34.54	236, 260, 330sh	0.257	37	124
flavanones	naringenin	10	280	34.31	289, 331sh	0.779	100	332
	naringenin-7-*O*-rutinoside	11	280	23.06	282, 328sh	0.865	91	302
cinnamic acids and derivatives	chlorogenic acid	12	320	7.16	231, 295sh, 327	1.340	19	62
	caffeic acid	13	320	10.39	296sh, 323	0.733	14	48
	p-coumaric acid	14	320	16.34	240, 298sh, 309	0.847	10	34
	ferulic acid	15	320	18.96	293sh, 323	1.152	44	148
	sinapic acid	16	320	19.66	291sh, 324	0.948	14	46
	m-coumaric acid	17	320	20.82	278, 324sh	0.227	28	92
	caffeic methyl ester	18	320	25.58	295sh, 325	0.492	20	68
	p-coumaric methyl ester	19	320	32.55	299sh, 311	1.999	14	46
	ferulic methyl ester	20	320	33.16	295sh, 324	3.246	16	52
	sinapic methyl ester	21	320	33.31	291sh, 325	1.602	17	58
	m-coumaric methyl ester	22	320	33.67	290, 323	0.536	22	72
flavones	apigenin	23	320	35.25	241. 267sh, 337	1.040	12	40
	luteolin	24	340	32.01	251, 264sh, 347	2.018	17	58
	apigenin-7-*O*-glucoside	25	340	24.36	240, 267sh, 337	0.369	38	128
flavon-3-ols	myricetin	26	360	24.85	265, 324sh, 370	0.934	34	114
	quercetin	27	360	31.36	265, 322sh, 373	1.132	13	44
	kaempferol	28	360	35.88	265, 322sh, 366	0.970	15	50
	quercetin-3-*O*-rutinoside	29	360	19.43	255, 267sh, 297sh, 354	1.448	52	172
	quercetin-3-*O*-glucoside	30	360	20.01	265sh, 298sh, 354	1.135	41	136
anthocyanins	delphinidin	31	520	12.71	272, 430sh, 531	2.664	12	40
	cyanidin	32	520	16.73	274, 430sh, 525	4.243	27	90
	pelargonidin	33	520	20.87	267, 327sh, 422, 514	1.172	22	72
	cyanidin-3-*O*-glucoside	34	520	8.48	280, 327sh, 517	6.392	30	100
	cyanidin-3-*O*-rutinoside	35	520	9.98	280, 329, 438sh, 519	2.845	58	194
	pelargonidin-3-*O*-glucoside	36	520	11.69	275, 330sh, 422sh, 504	3.264	32	108
other phenolics	ellagic acid	37	360	17.45	239, 253, 368	0.245	41	136
	resveratrol	38	320	26.72	241, 306sh, 321	1.140	19	62
	coniferyl alcohol	39	320	33.03	264, 293sh	0.120	92	306

[a] Recording wavelength (λ) used for the determination; [b] Retention time; [d] Calibration curves y = ax, where a is the slope, x is the peak area, and y is the concentration in μmol dm^{-3}; [e] Limits of detection (LOD); [f] Limits of quantification (LOQ). No.: numbers of peaks used in Fig. 1.

Table III. Recovery (%) of phenolic compounds added to leaf extracts of four plant species during different phases of extraction.

Plant	Internal standard	After extraction	After adding $CHCl_3$	Hydrolysis 40 min	Hydrolysis 100 min
P. zonale	M	98.6 ± 0.6	92.4 ± 1.6	87.1 ± 2.6	81.0 ± 2.9
	Q	99.8 ± 1.2	93.6 ± 2.1	91.1 ± 1.1	84.3 ± 0.9
	K	98.5 ± 0.4	68.3 ± 0.7	66.4 ± 2.1	61.2 ±1.8
	P	102.7 ± 1.7	99.8 ± 1.3	95.1 ± 3.7	93.5 ± 4.1
	CA	102.7 ± 0.8	97.4 ± 1.5	95.1 ± 2.1	88.5 ± 0.9
P. coleoides	M	103.7 ± 0.7	94.8 ± 2.5	86.0 ± 0.9	79.7 ± 2.4
	Q	98.9 ± 2.6	94.4 ± 1.0	92.7 ± 1.5	79.3 ± 2.1
	K	101.9 ± 2.0	71.3 ± 1.5	66.8 ± 1.4	62.4 ± 1.5
	P	97.4 ± 2.2	94.2 ± 1.0	92.4 ± 1.7	88.5 ± 1.7
	CA	99.5 ± 1.6	95.7 ± 1.1	87.9 ± 2.1	70.2 ± 3.2
T. platyphyllos	M	98.1 ± 2.2	95.5 ± 0.7	91.9 ± 2.1	81.9 ± 3.5
	Q	101.6 ± 1.1	92.9 ± 2.6	90.6 ± 0.3	84.2 ± 0.3
	K	98.7 ± 4.9	88.7 ± 2.0	84.1 ± 2.7	73.7 ± 1.5
	P	104.3 ± 2.8	97.7 ± 2.2	92.2 ± 1.7	87.6 ± 1.4
	CA	101.2 ± 3.4	98.8 ± 1.1	95.0 ± 1.7	85.3 ± 2.5
A. altissima	M	97.7± 1.3	96.6 ± 1.2	93.6 ± 0.9	88.1 ± 0.8
	Q	95.1 ± 3.9	91.7 ± 4.4	95.5 ± 0.5	78.3 ± 2.6
	K	95.6 ± 2.4	89.7 ± 3.2	86.1 ± 1.6	78.5 ± 1.2
	P	95.7 ± 1.4	99.8 ± 3.6	92.1 ± 1.5	87.9 ± 1.3
	CA	103.5 ± 1.6	96.0 ± 1.8	97.5 ± 0.7	89.1 ± 1.9

Aliquots of 20 μL of different standard phenolic solutions (all 1 mg mL^{-1}) were added to 150 mg of *P. zonale, P. coleoides, T. platyphyllos* and *A. altissima* leaf powder and then extracted and hydrolyzed as described in the Materials and Methods. M, myricetin; Q, quercetin; K, kaempferol; P, pelargonidin; CA, caffeic acid. Values are means ± SD, n = 3.

Differential patterns of phenolic compounds in the leaves of T. platyphyllos and A. altissima from forest park and high traffic areas. The presence of analytes in selected plant species was confirmed by comparing their retention times, spiking leaf extracts with pure standards and comparing the UV/VIS spectral characteristics with those of the standard compounds. The phenolic compounds previously identified in these plants (Toker *et al*. 2001, 2004; Kundu & Laskar 2010; Said *et al*. 2010) were also considered to identify the components in the present study. We found that the leaves from *A. altissima* had an approximately 16–fold higher concentration of total phenolic compounds by fresh weight than *T. platyphyllos* from the forest park area (Table IV). Among the hydroxycinnamates, the concentrations of *p*-coumaric acid, caffeic acid and caffeic methyl ester were seven, 160 and 237 times higher, respectively, in the leaves of *A. altissima* (Table IV). In the flavon-3-ols subclass, the quercetin and kaempferol concentrations were more than ten times higher in the leaves of *A. altissima,* while myricetin was only found in *T. platyphyllos* leaves. No flavones, luteolin or apigenin were seen in *T. platyphyllos* (Toker *et al*. 2001, 2004).

In the leaves of *A. altissima* from the heavy traffic area the most significant increase in the group of hydroxycinnamates was observed in chlorogenic acid derivative, caffeic acid and its methyl ester content compared with the trees from the forest park (Table IV). Additionally, among the flavonoids, the concentrations of luteolin and quercetin were significantly increased in the leaves of *A. altissima* plants from the heavy traffic area. On the other hand, the concentrations of all phenolic compounds were from two to five times higher in the leaves of *T. platyphyllos* collected at the high traffic location than in those collected from the park. Besides the accumulation of phenolics in general, we observed increased concentrations of catechol-type phenolics: (+)-catechin, myricetin, quercetin and anthocyanins derived from proanthocyanins, such as delphinidin and cyanidin in the leaves of *T. platyphyllos* in the high traffic area compared to those from the protected park (Table IV). There are already reports of the induction of phenolic compounds (quercetin and myricetin glycosides)

Table IV. Phenolic compounds in the leaves of *A. altissima* and *T. platyphyllos* under different urban conditions.

Phenolic compounds	*A. altissima* forest park	*A. altissima* heavy traffic area	*T. platyphyllos* forest park	*T. platyphyllos* heavy traffic area
hydroxycinnamates				
protocatechuic acid	30.356 ± 2.514	35.647 ± 3.564	3.481 ± 0.121	6.429 ± 0.324*
p-hydroxy benzoic acid	16.382 ± 1.322	17.748 ± 0.341	0.383 ± 0.024	0.878 ± 0.065*
syringic acid	43.749 ± 2.418	42.974 ± 2.245	2.079 ± 0.116	3.704 ± 0.213*
hydroxycinnamates				
chlorogenic acid	0.269 ± 0.007	0.288 ± 0.030	n.d.	n.d.
chlorogenic acid derivative[a]	1.117 ± 0.022	2.718 ± 0.251*	n.d.	n.d.
caffeic acid	2.127 ± 0.176	3.697 ± 0.119***	0.013 ± 0.002	0.053 ± 0.002**
p-coumaric acid	0.165 ± 0.010	0.254 ± 0.010**	0.024 ± 0.002	0.141 ± 0.012*
caffeic acid methyl ester	3.794 ± 0.366	6.515 ± 0.311***	0.015 ± 0.001	0.065 ± 0.007*
p-coumarate methyl ester	0.416 ± 0.024	0.561 ± 0.005	0.046 ± 0.003	0.191 ± 0.013*
flavan-3-ols				
(+)-catechin	6.928 0.567	4.808 ± 0.005*	0.287 ± 0.021	0.676 ± 0.046***
flavones				
luteolin	1.322 ± 0.055	2.768 ± 0.179**	n.d.	n.d.
apigenin	0.895 ± 0.013	1.157 ± 0.053	n.d.	n.d.
flavon-3-ols				
myricetin	n.d.	n.d.	0.010 ± 0.001	0.059 ± 0.004**
quercetin	3.011 ± 0.182	6.743 ± 0.079***	0.197 ± 0.016	0.376 ± 0.033*
kaempferol	1.280 ± 0.086	1.767 ± 0.105	0.102 ± 0.007	0.668 ± 0.048*
anthocyanins				
cyanidin	0.008 ± 0.001	0.013 ± 0.001*	0.012 ± 0.001	0.036 ± 0.002**
delphinidin	n.d.	n.d.	0.009 ± 0.001	0.073 ± 0.004**

[a] chlorogenic acid derivative: Rt = 14.6 min; spectral characteristics: 230, 294sh, 327 nm. Values labeled with * differ significantly from the leaf samples taken from unpolluted sites and locations in the sun ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$). n.d., non-detected. Values are shown in μmol per g_{Fw} and are means ± SE (n = 6).

in trees exposed to air pollution and heavy metals in urban conditions (Loponen *et al.* 1997; 1998; Michalak 2006).

CONCLUSIONS

We developed a rapid, efficient and accurate method for testing a variety of phenolic compounds, from the first step of homogenization, extraction, through purification, hydrolysis and separation to detection and quantification by HPLC. The main advantages of our method lie in its accuracy and reliability because it preserves the phenolic compounds' original chemical structures. The recovery was more than 95% after extraction, and still more than 84% after 40 min of hydrolysis. HPLC analysis enabled the simultaneous separation and identification of a broad range of phenolics, from the very polar such as gallic acid and anthocyanins, to the less polar luteolin and kaempferol. The broad range of analytes that could be separated and measured also included phenolic glycosides.

This method was used to identify and quantify soluble phenolic compounds in the leaves of two urban tree species, *A. altissima* and *T. platyphyllos*. Here, we revealed the presence of cyanidin glycosides in the leaves of *A. altissima*: to our knowledge, this is the first report of anthocyanins being detected in the leaves of this species. The presented method enables determination of species-specific phenolic profiles in the leaf tissue and contributes to monitoring of metabolic response under various environmental conditions.

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Botanica SERBICA

REZIME

Unapređena HPLC-DAD metoda za simultano određivanje fenolnih jedinjenja u listovima *Tilia Platyphyllos* i *Ailanthus Altissima*

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Fenolna jedinjenja predstavljaju jedne od najznačajnijih sekundarnih metabolita u biljkama i učestvuju u velikom broju fizioloških procesa. Tačno određivanje sadržaja fenolnih jedinjenja u biljkama je otežano zbog raznovrsnosti njihove hemijske strukture, podložnosti autooksidaciji, kao i antioksidantivne i prooksidativne sposobnosti. U ovom radu predstavljena je optimizovana procedura za ekstrakciju i hidrolizu fenolnih jedinjenja iz različitih biljnih vrsta uz potpuno očuvanje njihove hemijske strukture. Prikazana HPLC metoda omogućuje istovremeno razdvajanje i kvantifikaciju 39 jedinjenja iz različitih fenolnih grupa (benzoevih kiselina, hidroksi-cinamičnih kiselina, flavona, flavonola, flavanona, flavanola, izoflavona i antocijanidina). Efikasnost ekstrakcije određena dodavanjem standarda u uzorak lista, bila je veća od 95%, odnosno veća od 84% nakon potpune hidrolize glikozida. Metod je primenjen za analizu profila fenolnih jedinjenja u listovima dve vrste *Ailanthus altissima* (Mill.) Swingle i *Tilia platyphyllos* Scop. iz gradske park šume i ulice sa intenzivnim saobraćajem. Po prvi put prijavljeno je prisustvo cijanidin glikozida u listovima *A. altissima*. Rezultati ukazuju na povećanu akumulaciju fenolnih jedinjenja sa dve orto-hidroksi grupe, u odnosu na flavonoide sa monohidroksilnom supstitucijom u listovima obe vrste iz ulice sa intenzivnim saobraćajem.

Ključne reči: razvoj HPLC metode; antocijani; glikozidi flavonoida; fenolne kiseline; profil fenolnih jedinjenja; urbana sredina.

Appendix table I: The average concentrations of gases and particles measured in Kneza Milosa Street (Mostar) in August 2012 (www.sepa.gov.rs).

Location/parameter	Unit	Mostar
Sulphur dioxide	[μg/m^3]	8.5
Nitrogen dioxide	[μg/m^3]	57.6
Nitrogen monoxide	[μg/m^3]	37.2
Total nitrogen oxides	[μg/m^3]	78.7
Suspended particles PM10	[μg/m^3]	24.7
Suspended particles PM2.5	[μg/m^3]	48.4
Carbon monoxide	[μg/m^3]	-
Ozone	[μg/m^3]	-