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Bilin and Bilinone Chlorophyll Catabolite Content in the Hamamelidaceae Autumnal Leaves

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Abstract – In order to facilitate the quantification in autumnal Hamamelidaceae leaves, a HPLC method was used for the determination of two chlorophyll catabolites and their isomers: bilin-type (**1**) and bilinone-type (**2**) ones. The separation was done on a RP-C4 column with a gradient solvent system of 0.1% trifluoroacetic acid aqueous-methanol at the flow-rate of 0.2 mL/min and detected at 244 nm. The quantity of bilin-type (**1**) and bilinone-type (**2**) chlorophyll catabolite isomers from ten species of Hamamelidaceae autumnal leaves methanol extracts: *Corylopsis pauciflora, Corylopsis spicata, Forthergilla major, Hamamelis intermedia*, *Hamamelis japonicum, Hamamelis japonicum* var. *flavopurpurscens, Hamamelis virginiana, Parrotiopsis jacquemontiana*, *Parrotia persica* and *X Sycoparrotia semidecidua* were from 0.85 mg/g ~ 57.50 mg/g for bilin-type isomers (**1**) and 3.40 mg/g ~ 49.30 mg/g for bilinone-type isomers (**2**). The results obtained gave insight in quantitative bilin-type (**1**) and bilinone-type (**2**) chlorophyll catabolite composition of the Hamamelidaceae plant species autumnal leaves.

Keywords – Chlorophyll catabolites, HPLC, Hamamelidaceae, quantity

Introduction

Plant leaves are the most abundant chlorophyll producers. The chlorophylls are main fractions during the secondary metabolite isolation. They are a large family of biomolecules similar to each other and are the most abundant class of plant pigments.[1] The characterization of chlorophylls is well known.[2-5] The different chlorophyll catabolites are formed in leaves, in autumn with the begging of the exfoliation.[6] Among all the influences on chlorophyll catabolism, there are investigations indicating that the reactive nitrogen species (RNS), reactive oxygen species (ROS), the singlet oxygen species ($^1O_2$) and non-biological radicals take part in this biochemical process.[7-9] The chlorophyll oxidation may also occur through several other mechanistic pathways.[10] The chemical and enzymatic influences bring the exfoliates in a thin scaly plates to reveal cream, yellow, orange, red and light green leaves color patches.

There is lack in information on the quantitative strategies to profile the chlorophyll catabolites in autumnal plant leaves. The difficulties are mainly due to their short lifetime and a need for the HPLC coupled with mass spectrometry (MS) instrumentation for obtaining the reliable results. Autumnal leaves of Hamamelidaceae family plant species were chosen for the analysis due to the presence of several chlorophyll catabolites that are present in their autumnal leaves. Taking into consideration these photochemical compounds' characteristics, driven was a study aiming to determine the quantity of chlorophyll catabolites in the autumnal Hamamelidaceae leaves by HPLC, namely bilin-type (**1**) and bilinone-type (**2**) (Fig. 1).

Experimental

General experimental procedures – HPLC grade MeOH and water were purchased from Acros Organics™, (Geel, Belgium). Trifluoroacetic acid (TFA) used was Merck (Darmstadt, Germany). Other solvents and reagents were of analytical grade. The reference compounds were isolated from the plant material. The purities of isolated compounds were determined by TLC, ^{1}H NMR and ^{13}C NMR spectra. The internal standard uracil was purchased from the Merck (Darmstadt, Germany).

The leaves of *C. spicata* (*Cs*), *C. pauciflora* (*Cp*), *F. major* (*Fm*), *H. intermedia* (*Hi*), *H. japonicum* (*Hj*), *H. japonica* var. *flavopurpurescens* (*Hj* v. *f*), *H. virginiana* (*Hv*), *P. jacquemoutiana* (*Pj*), *P. persica* (*Pp*) and *X S.*

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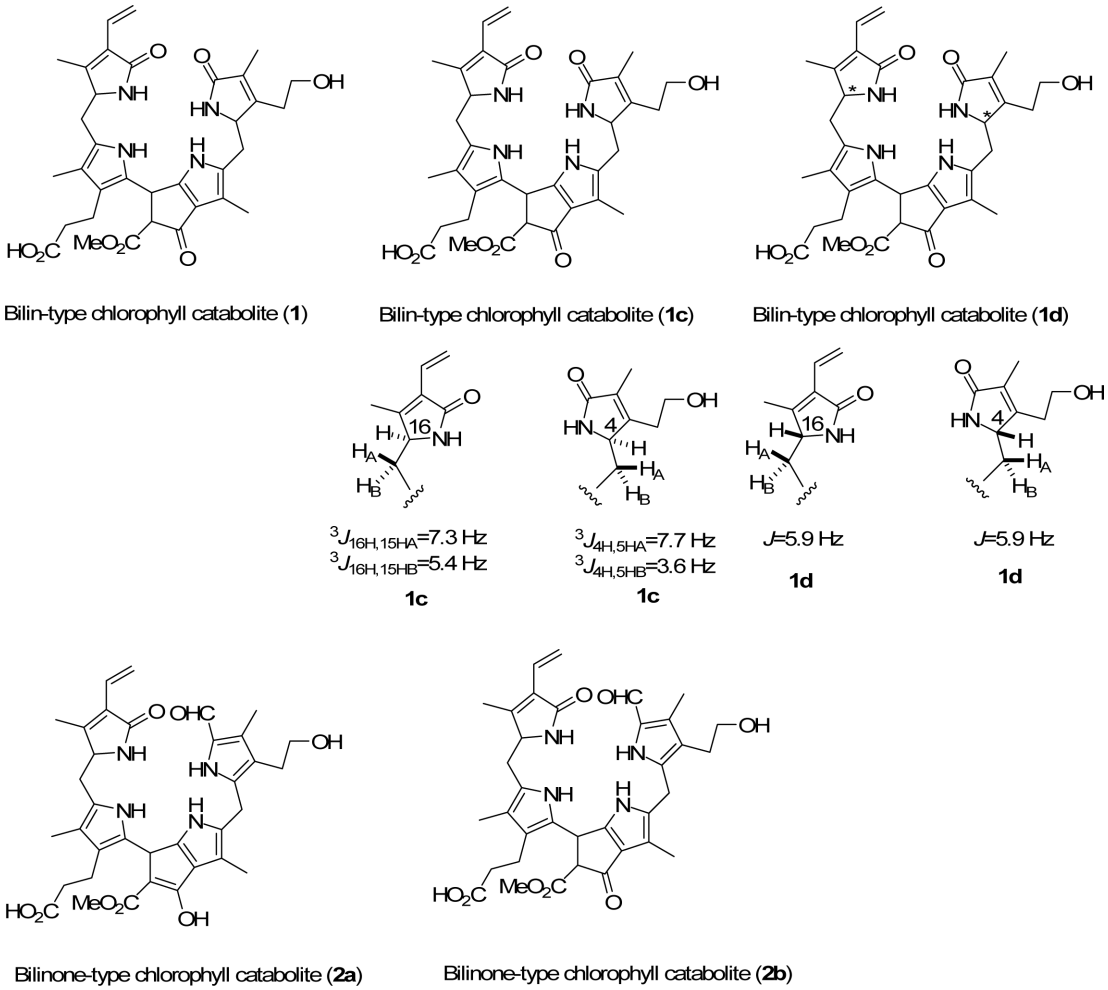


Fig. 1. Structure of bilin- (**1**) and bilinone- (**2**) chlorophyll catabolites.

semidecidua (*XSs*) were collected from local greenwood nurseries in October 2019.

HPLC apparatus and chromatographic conditions – The chromatographic system for quantitative analysis consisted of a separation module (Waters 2695, Milford, MA, USA), UV/Vis-PDA detector (Waters 2996, Milford, MA, USA), a mass spectrometer with an electrospray ionization (ESI) source (Esquire HCT, Bruker Daltonics, Bremen, Germany), and HyStar™ data processor (Bruker Daltonics, Bremen, Germany). The chromatographic separation of analyses was carried out on a Nucleosil 120-5 C4 (Macherey-Nagel, Dueren, Germany; 5 μm, 4.6 × 250 mm). Data was collected and analyzed using HyStar™ running under Windows NT™ (Microsoft, Redmond, USA). The mobile phase consisting of 0.1% TFA in water (A) and methanol (B) was run with gradient elution at a flow rate of 0.2 mL/min. The linear gradient elution was set as follows: 0 ~ 80 min; 10% B → 100% B. The injection volume was 10 μL. The UV absorption was monitored at 244 nm. The column temperature was maintained at 22 ºC. Quantification was conducted using an external standard method based on the peak area ratio of the analyte **1c** for bilin-type and **2b** for bilinone-type chlorophyll catabolites *versus* the amount of each analyte to create the calibration curves.

UV-Vis spectrophotometer – The UV-Vis spectra were recorded on Hach DR6000™ UV-Vis spectrophotometer (Loveland, Colorado, USA).

NMR spectrometer – The proton spectra were recorded on Bruker Avance 400 (Bruker, Rheinstetten, Germany).

Preparation of standard solutions – The standard solutions were prepared by dissolving separately 1.00 mg of each compound **1c** and **2b** in 1 mL methanol and appropriate concentrations were made. These solutions were stored away from light at 4 ºC.

Method validation – Two compounds **1c** and **2b** were each separately dissolved in methanol to the concentration of 1 mg/mL for the preparation of the stock solutions. The

Table 1. Calibration data for compounds **1c** and **2b** (n = 3)

Compound[a]	Regression equation (y = ax + b)[b]	R^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
1c	$y = 0.5442x - 1.0998$	0.9993	1.00 ~ 50.0	0.01	0.25
2b	$y = 0.2801x + 0.7522$	0.9990	1.00 ~ 50.0	0.01	0.25

[a]**1c** (bilin-type); **2b** (bilinone-type), [b]y is the peak area ratio, x is the corresponding injection concentration (μg/mL), a is the slope and b is the intercept of the regression line

stock solutions were then diluted to obtain different concentrations for each compound. The linearity was determined by plotting the peak area ratio *versus* concentrations of analytical standard.

The solution with the lowest concentration was diluted with methanol to yield a series of appropriate concentrations, and the limit of detection (LOD) and quantification (LOQ) under the chromatographic conditions were separately determined at signal-to-noise ratio (S/N) of about 3 and 10, respectively (Table 1).

Accuracy and precision – Recovery test was used to evaluate the accuracy of the assay. Analyzed were sets of five independent spiked at three different concentrations. Each sample was analyzed in triplicate. For comparison, a blank sample (not spiked with standard compounds) was prepared and analyzed. The average percentage recoveries were evaluated by calculating the ratio of detected amount *versus* added amount. The recovery rates were in the range 80.10 ~ 94.90%, and their RSD values were 1.6 ~ 2.4%.

Sample preparation – Samples (1.00 g) were weighted accurately and extracted with 10 mL methanol. The obtained extract was filtered to obtain the methanol sample solution. The 10 μL of the methanol sample solution containing the internal standard (uracil) was injected into the HPLC system in triplicate. The content of each compound was determined from the corresponding calibration curve.

Result and Discussion

The bilin- and bilinone-compounds analyzed **1**–**2** have the chromophore with the absorption at 244 nm (Fig. 2). According to the UV spectra of the compounds and their isomers in the range from 200 to 600 nm, 244 nm was set for monitoring bilin- and bilinone- chlorophyll catabolite isomers. The peaks of the bilin-**1** and bilinone-**2** chlorophyll catabolites, and their isomers, were assigned by spiking the samples with reference standards and comparison of their UV, mass spectra and retention times. The structure of **1c**, **1d**, **2a** and **2b** has been determined (Table 2 and Table 3). The structure of **1a**, **1b**, **1e**, **1f** and **1g** has not been determined, up to now. Their mass spectra with the m/z 633 is known, up to now (Fig. 3). The chromatograms of analyzed Hamamelidaceae autumnal leaves: *C. spicata* (*Cs*), *C. pauciflora* (*Cp*), *F. major* (*Fm*), *H. intermedia* (*Hi*), *H. japonicum* (*Hj*), *H. japonica* var. *flavopurpurescens* (*Hj* v. *f*), *H. virginiana* (*Hv*), *P. jacquemoutiana* (*Pj*), *P. persica* (*Pp*) and *X S. semidecidua* (*XSs*) were extracted at 244 nm and are shown in Fig. 4.

The established analytical method was applied to quantitatively analyze compounds **1**–**2** and their isomers in various samples of Hamamelidaceae species, using the regression equation. Their contents are summarized in Table 4.

The chlorophyll catabolite isomers **1** are bilin-type ones. The seven isomers of **1** were detected in leaves

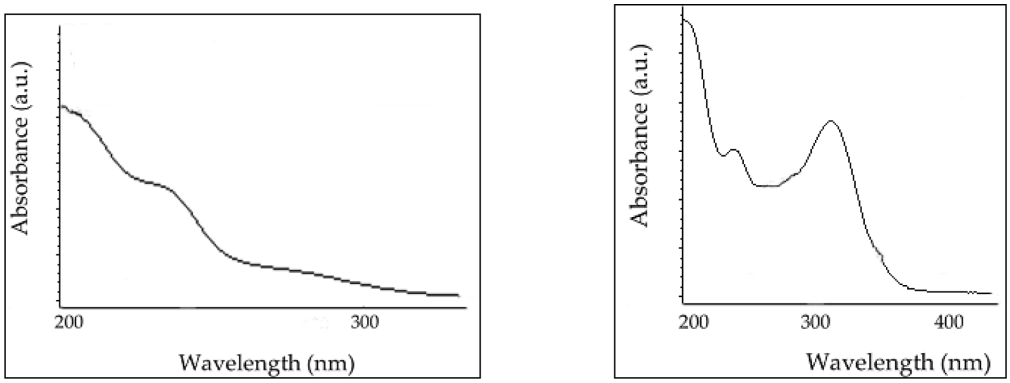


Fig. 2. UV spectra of the bilin- (**1**) (left) and bilinone- (**2**) (right) chlorophyll catabolites.

Table 2. ^{1}H NMR spectral data for compound **1c** and **1d** (400 MHz, CD_3OD)

Position	δ_H, multiplicity, J(Hz), **1c**	δ_H, multiplicity, J(Hz), **1d**
1		
2		
2^1	1.73 *s*	1.75 *s*
3		
3^1	2.49 *td* H_A (6.4, 13.9) 2.75 *ddd* H_B (6.4, 13.3)	2.52 *td* H_A (6.4, 13.9) 2.79 *ddd* H_B (6.4, 13.3)
3^2	3.68 *m* H_A and H_B (6.8, 13.4) (7.3, 13.9)	3.67-3.74 *m* H_A and H_B (6.8, 13.4) (7.3, 13.9)
4	4.34 *m* (4.5, 8.3)	4.34 *t* (5.9)
5	2.57 *dd* H_A (8.5, 14.9) 3.08 *dd* H_B (4.3, 14.9)	2.65 *dd* H_A (8.5, 14.9) 3.09 *dd* H_B (4.8, 14.9)
6		
7		
7^1	2.11 *s*	2.07 *s*
8		
8^1		
8^2	3.79 *d* (3.2)	n. d.
8^3		
8^4	3.75 *s*	3.75 *s*
9		
10	n. d.	n. d.
11		
12		
12^1	2.65 *t* H_A and H_B (7.7)	2.62 *t* H_A and H_B (7.7)
12^2	2.33 *dt* H_A (7.5, 15.6) 2.41 *dt* H_B (7.8, 15.5)	2.29 *dt* H_A (7.5, 15.6) 2.38 *dt* H_B (8.0, 15.9)
12^3		
13		
13^1	1.93 *s*	1.90 *s*
14		
15	2.60 *dd* H_A (5.9, 14.5) 2.91 *dd* H_B (5.2, 14.5)	2.71 *dd* H_A (5.9, 14.7) 2.91 *dd* H_B (5.4, 14.7)
16	4.09 *dd* (5.6, 7.9)	4.12 *t* (5.9)
17		
17^1	1.99 *s*	2.02 *s*
18		
18^1	6.43 *dd* H_X (11.7, 17.9)	6.42 *dd* H_X (11.4, 17.3)
18^2	5.34 *dd* H_A (2.3, 11.7) 6.07 *dd* H_M (2.4, 17.9)	5.33 *dd* H_A (2.3, 11.4) 6.06 *dd* H_M (2.3, 17.3)
19		

n. d. – not determined

Table 3. ^{1}H NMR spectral data for compound **2a** and **2b** (400 MHz, CD_3OD)

Position	δ_H, multiplicity, J(Hz), **2a**	δ_H, multiplicity, J(Hz), **2b**
1	4.02 *dd* (5.9, 8.2)	4.02 *dd* (5.9, 8.2)
2		
2^1	1.98 *s*	1.98 *s*
3		
3^1	6.46 *dd* (11.8, 17.7)	6.46 *dd* (11.8, 17.7)
3^2	5.38 *dd* (2.4, 11.7) H_A 6.12 *dd* (2.3, 18.2) H_M	5.38 *dd* (2.4, 11.7) H_A 6.12 *dd* (2.3, 18.2) H_M
4		
5	9.37 *s*	9.37 *s*
6		
7		
7^1	2.28 *s*	2.28 *s*
8		
8^1	2.58 *dd* (7.2, 13.2) H_A 2.66 *dd* (7.2, 13.2) H_B	2.58 *dd* (7.2, 13.2) H_A 2.66 *dd* (7.2, 13.2) H_B
8^2	3.53 *dd* (6.4, 13.6) H_A 3.58 *dd* (6.8, 12.7) H_B	3.53 *dd* (6.4, 13.6) H_A 3.58 *dd* (6.8, 12.7) H_B
9		
10	3.96 *d* (5.0)	3.96 *d* (5.0)
11		
12		
12^1	2.15 *s*	2.15 *s*
13		
13^1		
13^2		3.86 *d* (7.7)
13^3		
13^4	3.79 *s*	3.79 *s*
14		
15		4.65 *d* (6.3)
16		
17		
17^1	2.36 *dd* (6.5, 14.4) H_A 2.65 *dd*(6.6, 14.5) H_B	2.36 *dd* (6.5, 14.4) H_A 2.65 *dd*(6.6, 14.5) H_B
17^2	2.34 *dd* (7.3, 14.1) H_A 2.39 *dd* (7.7, 14.0) H_B	2.34 *dd* (7.3, 14.1) H_A 2.39 *dd* (7.7, 14.0) H_B
17^3		
18		
18^1	1.91 *s*	1.91 *s*
19		
20	2.88 *dd* (4.9, 15.0) H_A 2.50 *dd* (5.3, 15.0) H_B	2.88 *dd* (4.9, 15.0) H_A 2.50 *dd* (5.3, 15.0) H_B

extracts. Two isomers of the bilinone-type chlorophyll catabolite **2** have been quantified. Considering the quantitative profile, the highest total chlorophyll catabolite content was determined in *Hv* extract (91.08 μg/g) and the lowest content had *Cs* (22.08 μg/g) (Table 4). The most abundant catabolite in *Cp* extract was **1b** (49.3 μg/g). The **1b** catabolite in *Fm* extract (0.85 μg/g) was with the lowest quantity detected. In *Fm* extract the isomer **1f** was 10% more abundant than the **1g**, with quantities of 4.68 μg/g and 4.25 μg/g, respectively. The isomer **1d** had a

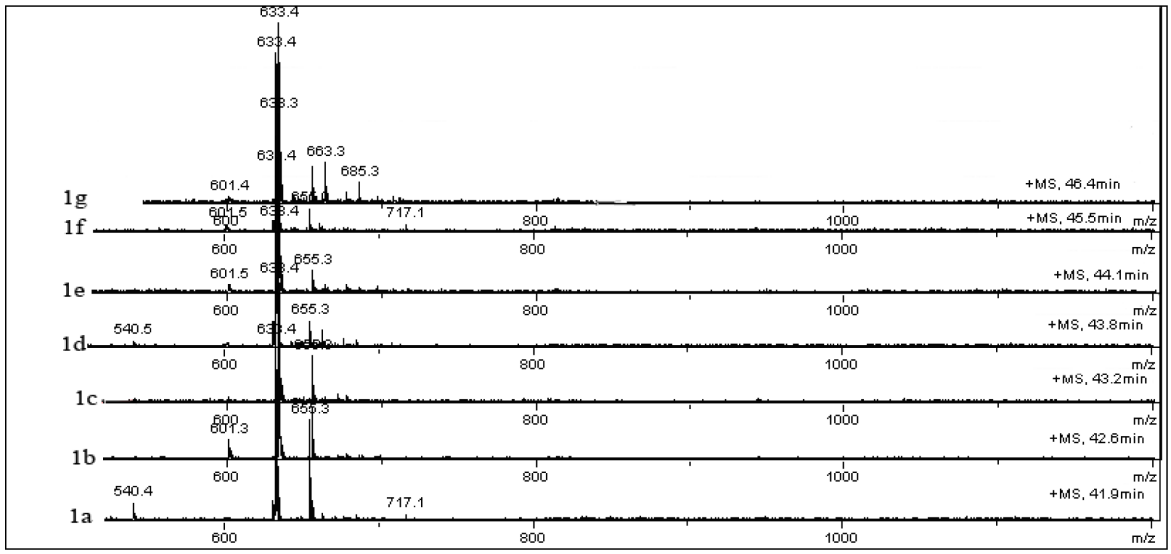


Fig. 3. Mass spectra of bilin chlorophyll catabolite isomers (**1**) detected in the autumnal Hamamelidaceae leaves.

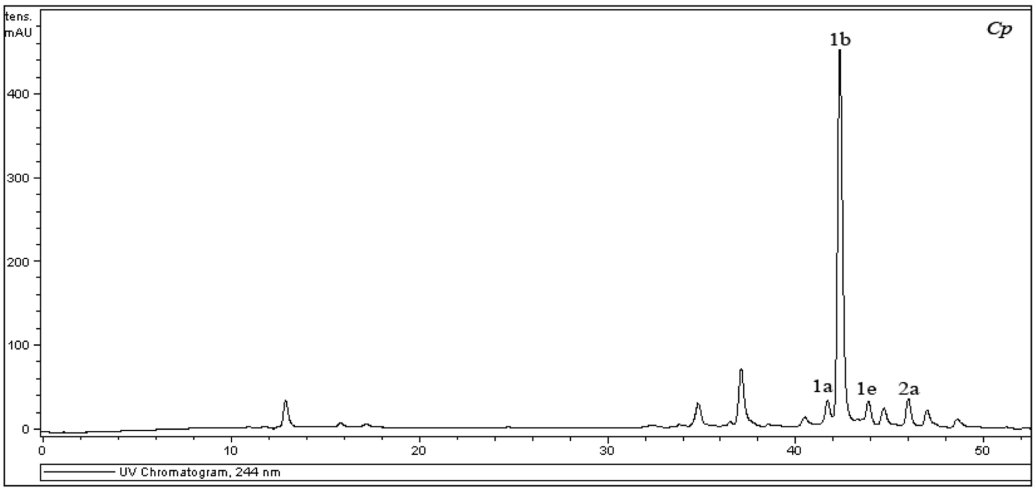
Table 4. Contents of bilin- (**1**) and bilinone- (**2**) chlorophyll catabolites in samples of autumnal Hamamelidaceae leaves (n = 3)

Sample	Content (μg/g)								
	1a	1b	1c	1d	1e	1f	1g	2a	2b
Cp	3.40 ± 0.2	49.30 ± 0.1	-	-	2.55 ± 0.3	-	-	4.25 ± 0.10	-
Cs	5.10 ± 0.1	4.23 ± 0.1	-	-	5.95 ± 0.1	-	-	6.80 ± 0.15	-
Fm	-	0.85 ± 0.1	-	11.90 ± 0.1	-	4.68 ± 0.1	4.25 ± 0.1	-	49.30 ± 0.1
Hi	-	3.41 ± 0.2	4.22 ± 0.2	-	27.95 ± 0.1	3.45 ± 0.1	8.52 ± 0.1	-	5.17 ± 0.1
Hj	-	4.02 ± 0.2	4.85 ± 0.2	12.11 ± 0.1	46.30 ± 0.1	8.78 ± 0.1	3.20 ± 0.3	-	7.27 ± 0.2
Hj v. f	-	-	6.88 ± 0.2	5.35 ± 0.1	44.30 ± 0.1	10.71 ± 0.1	3.06 ± 0.2	-	12.75 ± 0.1
Hv	-	7.01 ± 0.2	5.10 ± 0.1	57.50 ± 0.1	1.87 ± 0.1	7.50 ± 0.2	7.05 ± 0.2	-	5.05 ± 0.2
Pj	-	9.50 ± 0.1	-	-	-	6.46 ± 0.1	-	46.84 ± 0.1	5.32 ± 0.2
Pp	-	-	48.60 ± 0.1	-	2.55 ± 0.1	0.85 ± 0.1	0.85 ± 0.1	-	3.40 ± 0.2
X Ss	-	-	48.50 ± 0.1	-	-	-	-	-	-

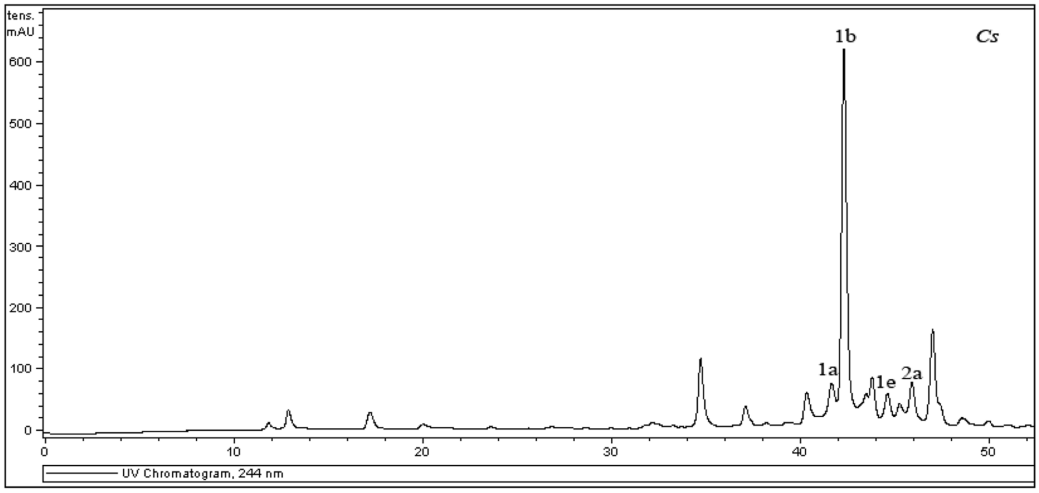
higher quantity and it was 11.9 μg/g. The **2b** catabolite exhibited the value of 49.3 μg/g showing the highest content value in that specie. In all the species analyzed that was the highest **2b** value. Seven chlorophyll catabatolites were identified in *Hi* extract and were: **1b**, **1c**, **1e**, **1f**, **1g** and **2b**. Seven chlorophyll catabolites were identified in *Hj* and *Hv* being **1b**, **1c**, **1d**, **1e**, **1f**, **1g** and **2b**. In *Hj* the catabolite **1** profile had six different isomers. Of those six isomers, the **1e** represented 46.30 μg/g, the **1d** was present in 12.11 μg/g and **1f** quantity was 8.78 μg/g. The isomers **1b**, **1c** and **1g** were present in less than 5 μg/g. The chlorophyll catabolite in *X Ss* **1c** was present in the quantity of 48.50 μg/g. The *Pp* extract had four bilin-type catabolites: **1c**, **1e**, **1f** and **1g**. Among them, the catabolite **1c** was the major one with 48.60 μg/g. The major catabolite detected in *Pj* extract was catabolite **2a** (46.84 μg/g) and then **1b** (9.50 μg/g). Other major peaks in *Pj* plant leaves methanol extract chromatogram were identified and were: **1f** (6.46 μg/g) and **2b** (5.32 μg/g). The isomers **2a** and **2b** were detected and were in ratio 8.8:1.

In this study a characterization of the chlorophyll catabolite **1** and **2** isomers in ten Hamamelidaceae autumnal leaves methanol extracts was done by HPLC. The analytical characterization revealed the presence of seven bilin-type chlorophyll catabolite isomers (**1**) and two bilinone-type (**2**) ones. Considering the quantitative profile, the highest total catabolite content was determined in the *Hv* extract (91.08 μg/g) and *Hj* extract (86.53 μg/g). The quantification of each catabolite was species-specific.

Cp



Cs



Fm

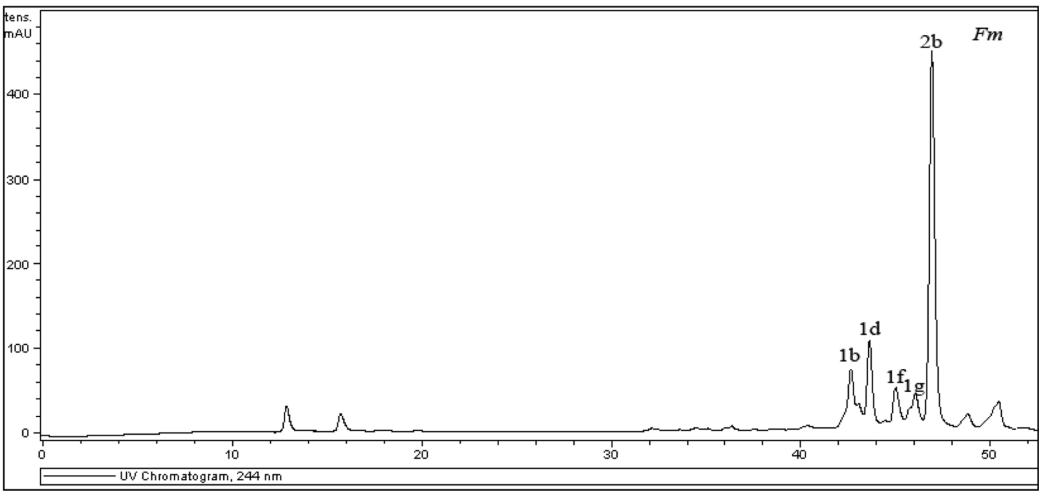
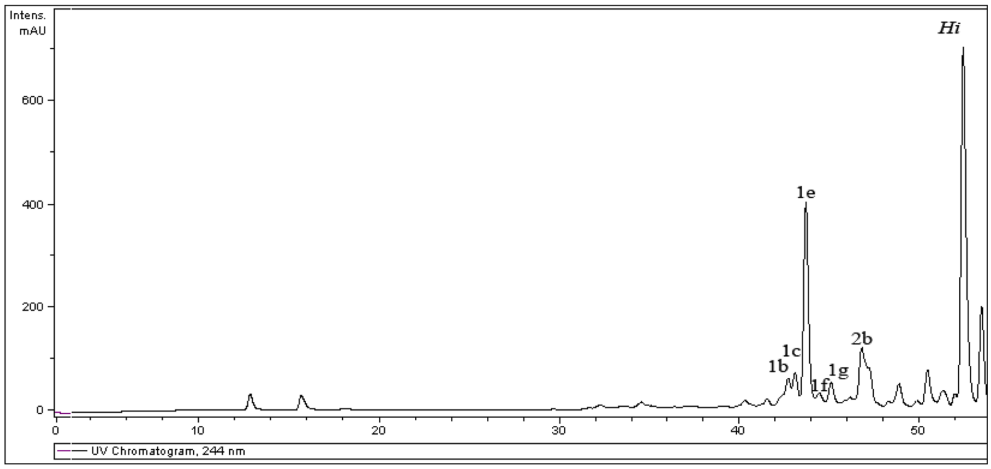
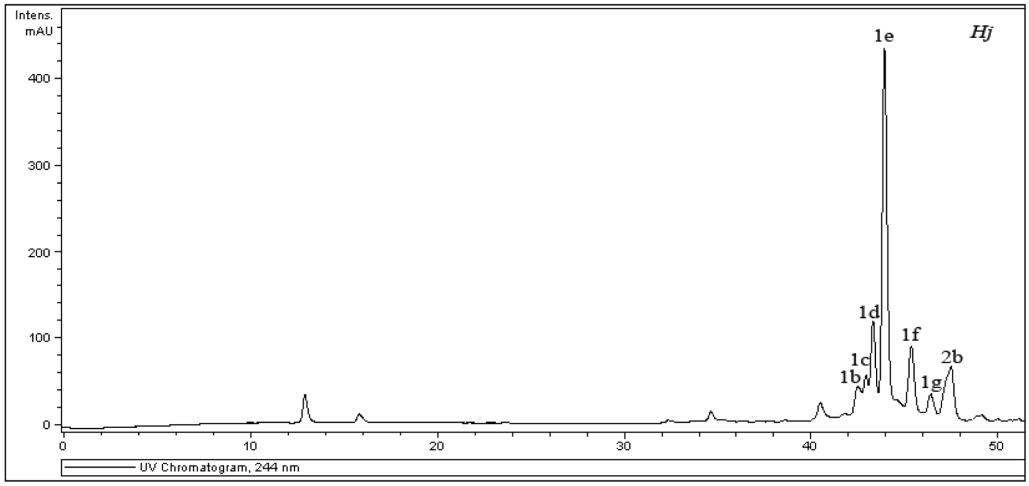


Fig. 4. HPLC chromatogram of bilin- (**1**) and bilinone- (**2**) type chlorophyll catabolites isolated from the autumnal Hamamelidaceae leaves.

Hi



Hj



Hj var. *f*

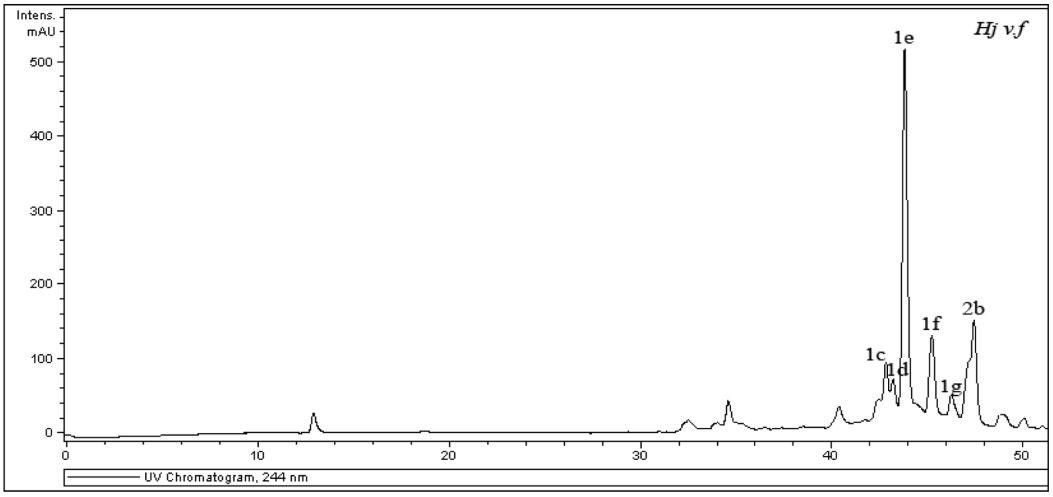
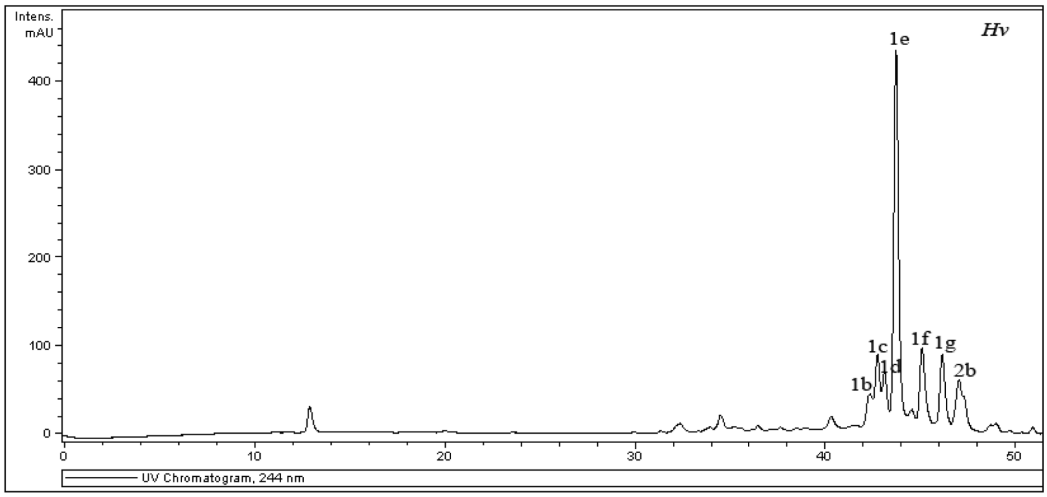
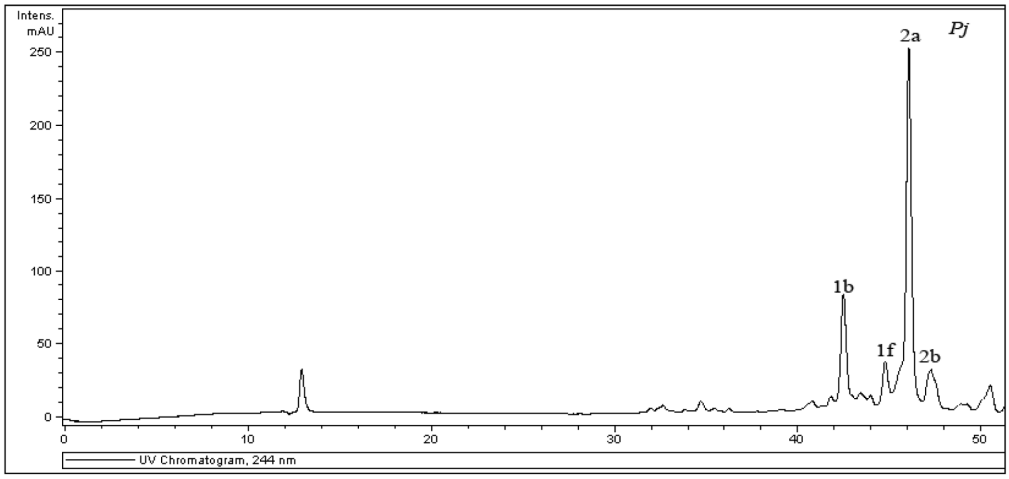


Fig. 4. continued

Hv



Pj



Pp

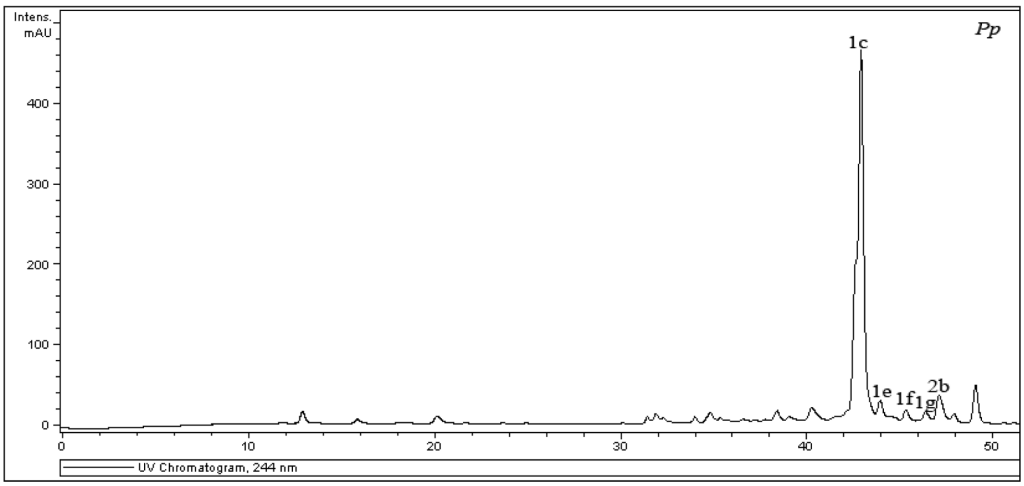


Fig. 4. continued

XSs

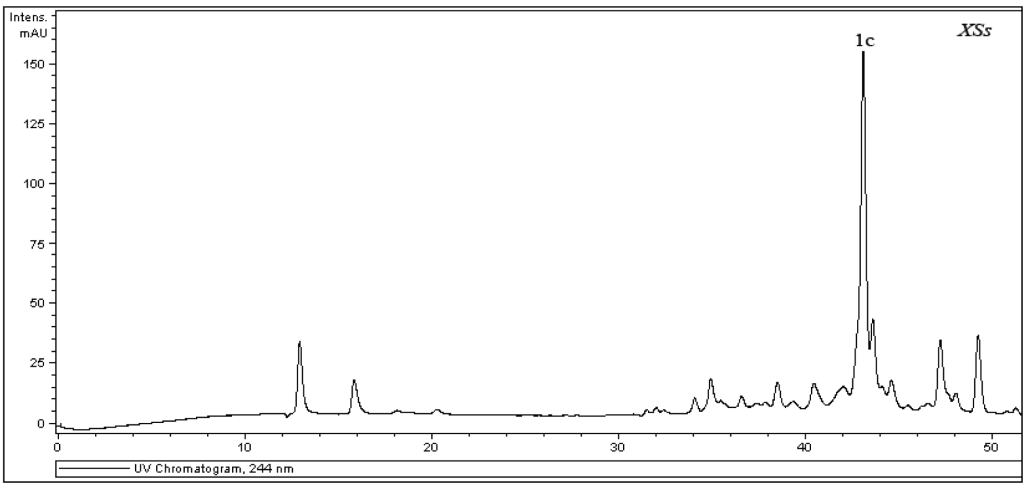


Fig. 4. continued

Among the catabolites identified, relevant catabolic quantity had **1b** in *Cp*, **1c** in *Pp* and *X Ss*, **1d** in *Hv*, **2a** in *Pj* and **2b** in *Fm*.

In this study, a reliable analytical method for simultaneous quantification of the bilin-type chlorophyll catabolite isomers (**1**) and two bilinone-type (**2**) ones was developed using HPLC. Separation was achieved on a Macherey-Nagel Nucleosil 120-5 C4 column (5 μm, 250 × 4.6 mm i.d.) with a gradient solvent system of 0.1% trifluoroacetic acid aqueous-methanol, at a flow rate of 0.2 mL/min, and detected at 244 nm. The developed assay has been applied successfully to quantify the two compounds, seven isomers of **1** and two isomers of **2**, in ten autumnal leaves methanol extract of the Hamamelidaceae family. The variation in contents of bilin-(**1**) and bilinone-(**2**) chlorophyll catabolite compounds is influenced by the source of nutrients, morphological and cytological characteristics and the composition of genes and enzymes specific in each species.[11] It should be mentioned that in the Hamamelidaceae autumnal leaves extracts chlorophyll catabolites vary due to the time of harvesting. Therefore, the simultaneous determination of bilin-(**1**) and bilinone-(**2**) chlorophyll catabolites provides a valuable view for detecting and quantifying these natural pigments.

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