CHLOROPHYLL CATABOLISM IN *HYDRANGEA ASPERA* AUTUMNAL LEAVES

NINA DJAPIC*

"Mihajlo Pupin", University of Novi Sad, Technical Faculty Djure Djakovica bb, 23000 Zrenjanin, Serbia

(Received January 26, 2013)

The *Hydrangea aspera* autumnal leaves were screened for the presence of chlorophyll catabolites. The chlorophyll catabolites found in *Hydrangea aspera* autumnal leaves extracts were analyzed by liquid chromatography/mass spectrometry. The spectra obtained revealed the presence of non-glycosylated and glycosylated chlorophyll catabolites. The results obtained permitted the proposition of the chlorophyll catabolic pathways in *Hydrangea aspera* autumnal leaves.

Key words: *Hydrangea aspera*, LC/MS, chlorophyll catabolites.

INTRODUCTION

In the higher plant cell chlorophyll catabolism consists of a great number of steps. The loss of the phytol side chain induces the formation of the chlorophyllide *a* (1, 2, 3). After the loss of magnesium from the core of the chlorophyllide *a* the pheophorbide *a* (**1**) is formed (Fig. 1) (4). The so-called "north" opening of the pheophorbide *a* (**1**) ring is mechanistically postulated and yields the 4,5-dioxo-*seco*–pheophorbide *a* chlorophyll catabolite (**2**) (Fig. 1).

Once the pheophorbide *a* ring is opened, the subsequent tautomerization induces the loss of fluorescence. Chlorophyll catabolism proceeds further.

The reverse phase liquid chromatography-mass spectrometry (RP-LC-MS) methods have been used in the identification of the *Hydrangea aspera* chlorophyll catabolites. The hyphenated techniques provided the information on the *m/z* of the *Hydrangea aspera* chlorophyll catabolites and allowed their structural determination by their molecular mass (5).

* Corresponding author (Email: djapic@tfzr.uns.ac.rs)

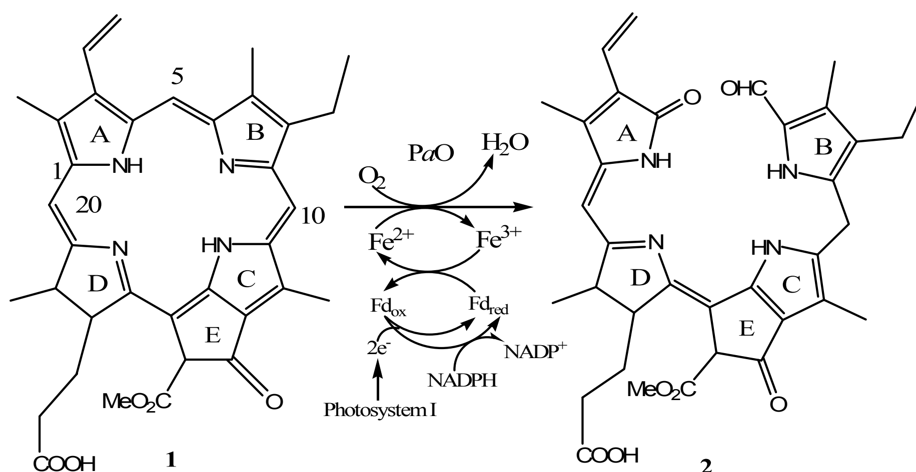


Fig. 1. – The so-called "north" opening of the pheophorbide *a* (**1**) ring by the enzyme pheophorbide *a* oxygenase (P*a*O) induces the formation of the 4,5-dioxo-*seco*-pheophorbide *a* (**2**).

MATERIALS AND METHODS

Hydrangea aspera D. Don ssp. Sargentiana E. M. McClint autumnal leaves (30 g dry weight, 40 g "fresh" weight) were chilled with liquid nitrogen, ground and homogenized in a blender with 0.5 dm^3 methanol, at room temperature, for 10 minutes. After centrifugation, the methanol extract was filtered and partitioned between hexane and methanol. Water was added to the methanol phase. The obtained volume was divided into two parts. From one part, *Hydrangea aspera* chlorophyll catabolites were extracted with dichloromethane from the methanol-aqueous phase. The evaporation of dichloromethane ($t<40^oC$) yielded 22.42 mg. From the other part, the *Hydrangea aspera* chlorophyll catabolites were extracted with ethyl acetate from the methanol-aqueous phase. The evaporation of ethyl acetate ($t<40^oC$) yielded 19.73 mg. The extracts obtained were dissolved in methanol and subjected to the liquid chromatography mass spectrometry (LC-MS) analysis. Methanol and water used for the LC separation were HPLC grade (Acros Organics, Geel, Belgium) and trifluoroacetic acid (TFA) was reagent grade (Fluka, Buch, Switzerland). The LC/UV/ electrospray ionization (ESI) – MS analyses were performed on Waters 2695 Separations Module (Milford, MA, USA) coupled to a Waters 2996 photodiode array (PDA) UV-Vis detector and connected to Bruker Daltonics esquire high capacity ion trap (HCT) (Bruker Daltonik, GmbH, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Nitrogen produced by nitrogen generator (Domnick Hunter Group plc, Durham, England) was used as nebulizer (20 psi) and drying gas (9 L min^{-1} at 320^0C) in the ESI experiments. The ESI detection was done in the positive ion mode with the target

mass of 900 *m/z*. The capillary voltage in a ramp ranged from 4.5 to 1.5 kV. Data were acquired by HyStar™ and processed by Bruker Daltonics Data Analysis running under Windows NT™ (Microsoft, Redmond, USA). The LC separations were carried on the reverse phase (RP) column with the stationary phase EC 250x4 mm Nucleosil® 120-5 C_4 column together with CC 8x4 mm Nucleosil® 120-5 C_4 precolumn (Macherey-Nagel, Oesingen, Switzerland). The injection volume was 10 μL *via* autosampler injection and in every sample, 10 μL of uracil (0.01 mg mL^{-1}) was dissolved. The temperature of the column oven was 298 K. The mobile phase consisted of methanol and 0.1% TFA in water. The proportion of methanol was increased linearly from 10% to 100% in 80 minutes and in the last 10 minutes the elution was with 100% methanol. The flow rate was 0.2 mL min^{-1}. After each separation, the column was re-equilibrated linearly from 100% methanol to 90% water (0.1% TFA):10% methanol in 10 minutes and additionally 5 minutes at 90% water (0.1% TFA):10% methanol. Data were acquired by HyStar™ and processed by Bruker Daltonics Data Analysis running under Windows NT™ (Microsoft, Redmond, USA).

The limit of detection (LOD) and the limit of quantization (LOQ) were determined on the basis of the signal to noise ratio. The LOD and the LOQ were calculated by the method based on the standard deviation (S. D.) of the response and the slope (S) of the calibration curve (6, 7).

RESULTS

The LC–MS analysis of the *Hydrangea aspera* autumnal leaves dichloromethane and ethyl acetate extracts were subjected on the RP–C_4 analytical column (5). The chromatograms obtained revealed the presence of the chlorophyll catabolites depicted in Fig. 2.

DISCUSSION

The extracts of *Hydrangea aspera* autumnal leaves were analyzed on the RP–C_4 analytical column by LC–MS. The structure of the chlorophyll catabolites was determined by their molecular mass (5, 8). The chlorophyll catabolites identified permitted the proposition of the chlorophyll catabolism in the *Hydrangea aspera* autumnal leaves (Figs. 3 and 4). The chlorophyll catabolism continues from the 4, 5-dioxo-*seco*-pheophorbide *a* (**2**) to the formation of the hydroxylated ethyl side chain chlorophyll catabolite (**4**). The chlorophyll catabolite **4** after the oxidation of the vinyl group forms the dihydroxylated chlorophyll catabolite **6**.

After the acidic catalyzed tautomerization, chlorophyll catabolites **2**, **4** and **6** form the thermodynamically more stable catabolites **2’**, **4’** and **6’** (9). The reduction of the thermodynamically more stable compounds (**2’**, **4’**, **6’**) proceeds

via the reduction of the "western" methene bridge, in the presence of ferredoxin, forming the chlorophyll catabolites (**9**, **3**, **5**). Further, chlorophyll catabolites were, up to now, not detected, due to the absence of the chromophore that can absorb the UV light.

The chlorophyll catabolites with the *m/z* 805.4 (**8**) and *m/z* 807.3 (**7**) indicated the presence of the other chlorophyll catabolic pathway in the *Hydrangea aspera* autumnal leaves. The other chlorophyll catabolic pathway proceeds from the hydroxylated chlorophyll catabolite at the $C8^2$ position (**4**) and its glycosylation at the $C8^2$ position (**8**) (10). The glycosyl bond is most probably introduced by glycosyltransferase. The chlorophyll catabolites **4** and **8** form a thermodynamically stable aromatic ring D under the acidic conditions (**4'** and **8'**, respectively). The thermodynamically stable chlorophyll catabolites in the presence of ferredoxin undergo the reduction of the "western" methene bridge forming the chlorophyll catabolites **3** and **7**, respectively (Fig. 4).

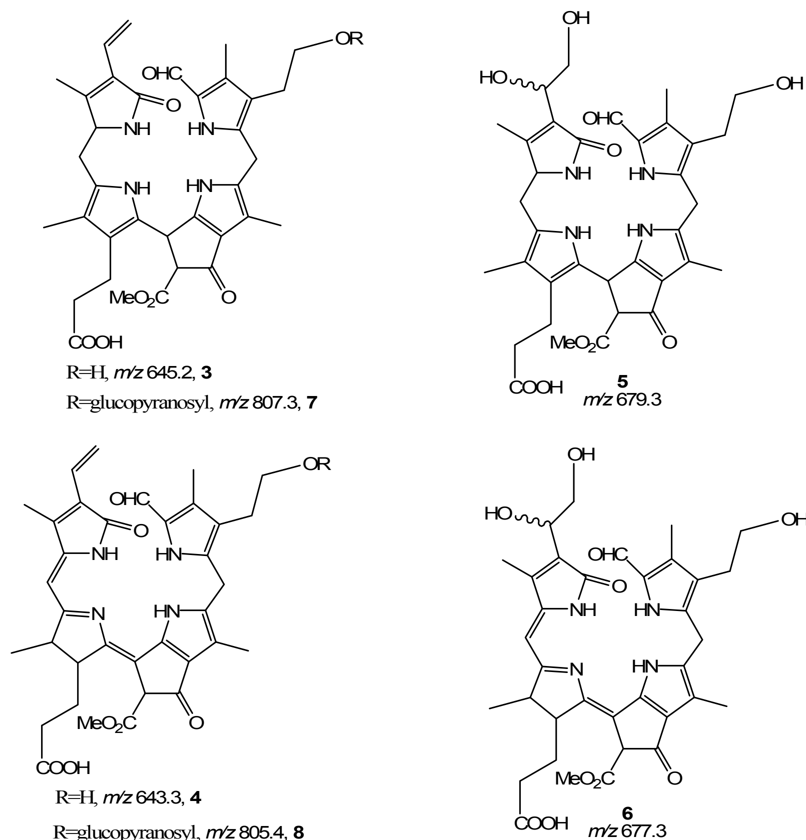


Fig. 2. – Chlorophyll catabolites present in *Hydrangea aspera* autumnal leaves dichloromethane and ethyl acetate extracts

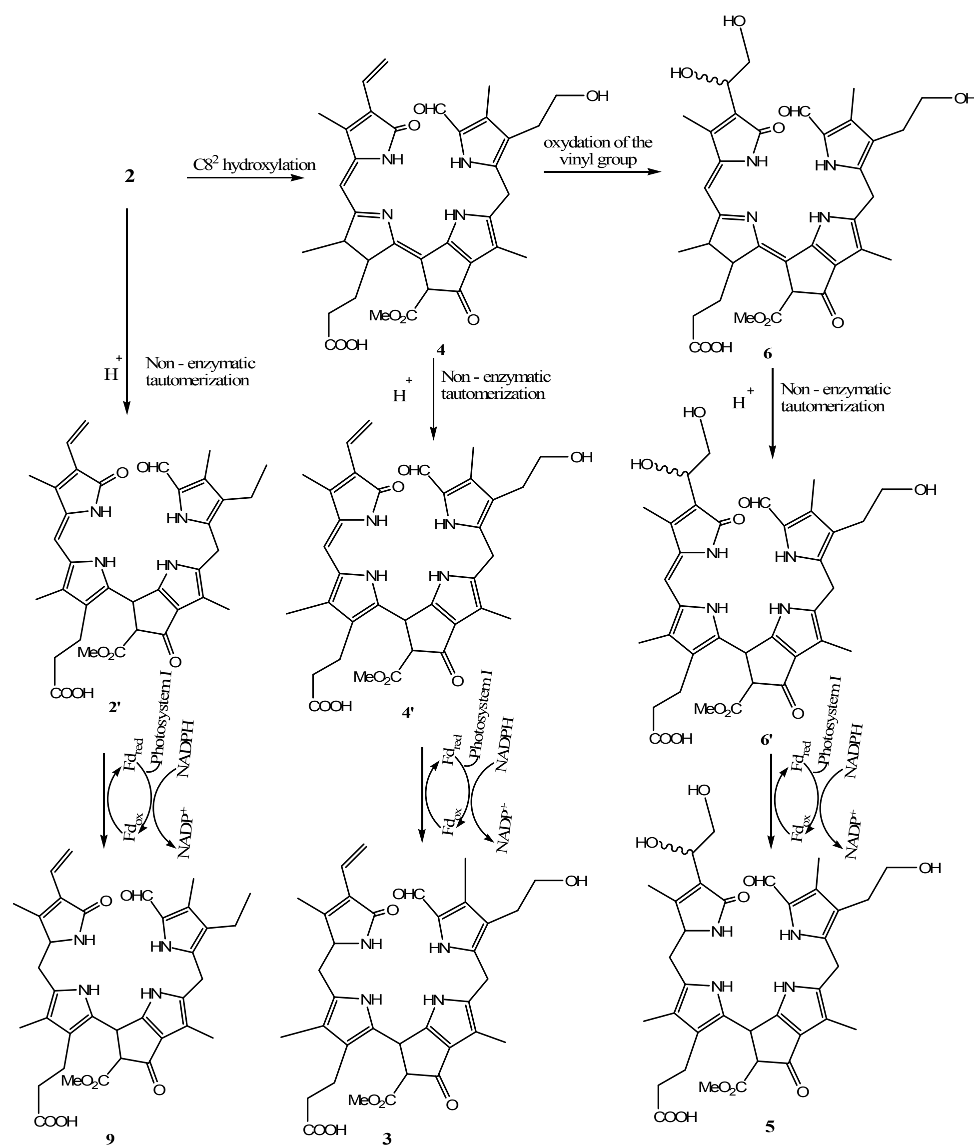
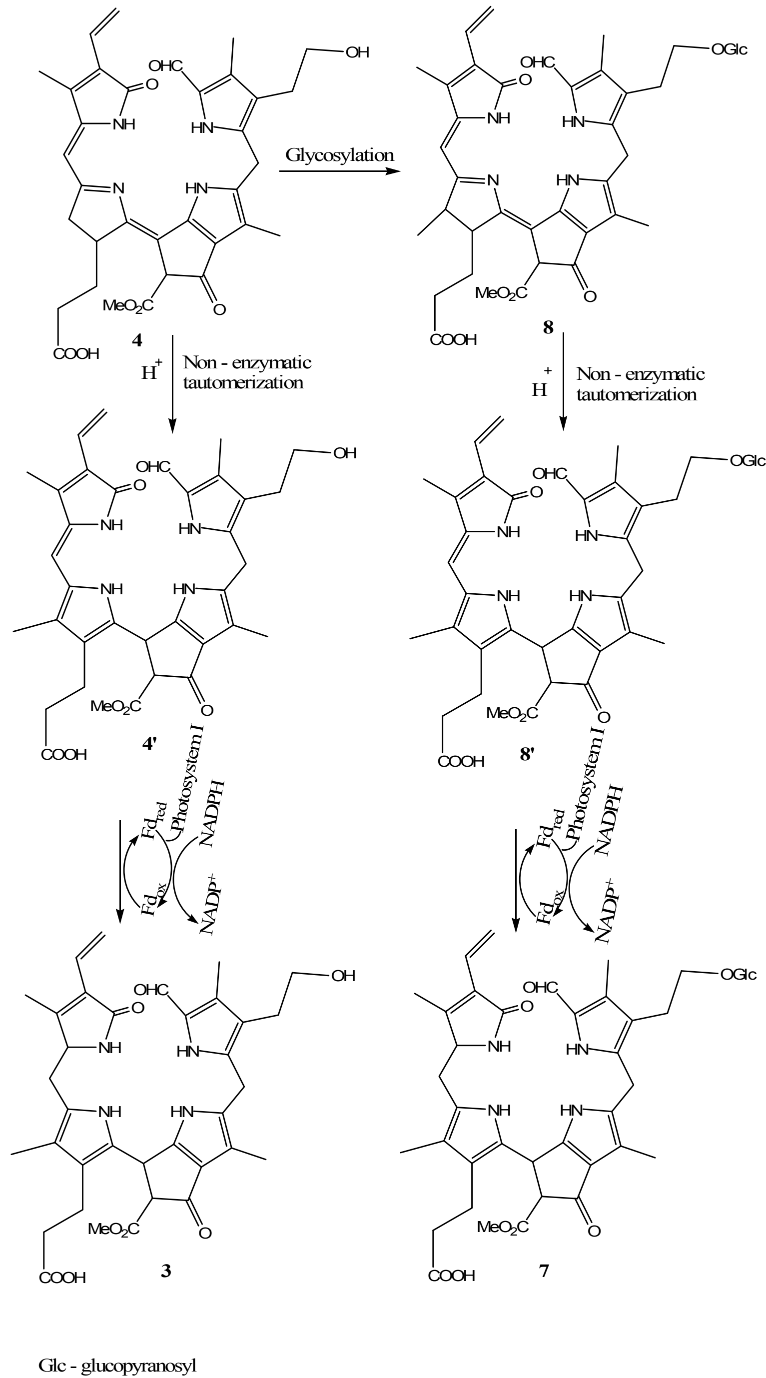


Fig. 3. – One chlorophyll catabolic pathway in *Hydrangea aspera* autumnal leaves.



Glc - glucopyranosyl

Fig. 4. – The other chlorophyll catabolic pathway in *Hydrangea aspera* autumnal leaves.

The further detection of the chlorophyll catabolites present in *Hydrangea aspera* autumnal leaves will extend the chlorophyll catabolic pathways that are known up to now.

The technique used works well for the chlorophyll catabolite analytes retained on the RP stationary phase. The LOD for the chlorophyll catabolites **3**, **5** and **7** was found at the level of 8.10 ng mL^{-1}, 20.5 ng mL^{-1} and 8.70 ng mL^{-1}, respectively. The LOQ was at the levels of 24.5 ng mL^{-1}, 62.0 ng mL^{-1} and 26.2 ng mL^{-1} for the chlorophyll catabolites **3**, **5** and **7**, respectively. The values obtained indicated that the LC-MS method used is sensitive in the analysis of the chlorophyll catabolites.

CONCLUSIONS

The extraction of *Hydrangea aspera* chlorophyll catabolites from the autumnal leaves' methanol extract with dichloromethane and ethyl acetate revealed the presence of nine chlorophyll catabolites. The identified *Hydrangea aspera* chlorophyll catabolites permitted the construction of the chlorophyll catabolic pathways. Further investigations on *Hydrangea aspera* chlorophyll catabolites are desirable. There is still much to be discovered about chlorophyll catabolism at the biochemical and genetic levels.

REFERENCES

1. Brown SB, Houghton JD, Hendry GAF, *Chlorophyll breakdown*, in: **Chlorophylls**, Scheer H, ed, CRC Press, Boca Raton, FL, 1991, pp. 466–476.
2. Willows RD, *Biosynthesis of chlorophylls from protoporphyrin IX*, ***Nat. Prod. Rep.***, **20**, 327–341 (2003).
3. Ruediger W, *Biosynthesis of chlorophyll b and the chlorophyll cycle*, ***Photosynthesis Research***, **74**, 187–193 (2002).
4. Shioi Y, Watanabe K, Takamiya K, *Enzymatic conversion of Pheophorbide a to the precursor of Pyropheophorbide a in leaves of Chenopodium album*, ***Plant and Cell Physiol.***, **37**, 1143–1149 (1996).
5. Djapic N, *Thermodynamic study of Hydrangea aspera chlorophyll catabolites by reverse phase liquid chromatography*, ***Studia UBB Chemia***, **LVIII**, **2**, 43–52 (2013).
6. Ardrey RE, *Liquid Chromatography*, in: **Liquid Chromatography – Mass Spectrometry: An Introduction**, John Wiley & Sons Ltd, England, 2003, pp. 25–26.
7. Ardrey RE, *Applications of High Performance Liquid Chromatography – Mass Spectrometry*, in: **Liquid Chromatography – Mass Spectrometry: An Introduction**, John Wiley & Sons Ltd, England, 2003, pp. 228–230.
8. Djapic N, Djuric A, Pavlovic A, *Chlorophyll biodegradation in Vitis vinifera var. Pinot noir autumnal leaves*, ***Research Journal of Agricultural Sciences***, **41**, 256–260 (2009).
9. Đapić N, *Behaviour of Fothergilla gardenii chlorophyll catabolite under acidic conditions*, ***Kragujevac J. Sci.***, **34**, 79–85 (2012).
10. Berghold J, Eichmueller C, Hoertensteiner S, Kraeutler B, *Chlorophyll Breakdown in Tobacco: On the Structure of Two Nonfluorescent Chlorophyll Catabolites*, ***Chemistry & Biodiversity***, **1**, 657–668 (2004).