STUDIA UBB CHEMIA, LVIII, 2, 2013 (p. 43 – 52)
(RECOMMENDED CITATION)

# THERMODYNAMIC STUDY OF *HYDRANGEA ASPERA* CHLOROPHYLL CATABOLITES BY REVERSE PHASE LIQUID CHROMATOGRAPHY

**NINA DJAPIC**[a]

**ABSTRACT.** The *Hydrangea aspera* chlorophyll catabolites present in autumnal leaves were investigated. The thermodynamic study of the *Hydrangea aspera* chlorophyll catabolites was done using reversed phase liquid chromatography on the $C_4$ and $C_8$ analytical columns with water (acidified):methanol mobile phase in combination with ultraviolet detection and electrospray ionization mass spectrometry identification. The retention behaviors of *Hydrangea aspera* chlorophyll catabolites over a temperature range of 278-318 K were investigated. The data obtained permitted the construction of the van't Hoff plots. The stationary phase composition influences the thermodynamic retention of the *Hydrangea aspera* chlorophyll catabolites.

***Keywords:*** *Hydrangea aspera; chlorophyll catabolites; Liquid Chromatography-Mass Spectrometry; van't Hoff plot*

## INTRODUCTION

The chlorophyll catabolism consists of a great number of steps and, up to know, chlorophyll catabolites that have a chromophore that can absorb the ultraviolet-visible (UV-Vis) light are known. In the plant cell, every step is coordinated, highly regulated and most steps are enzymatically catalyzed. The chlorophyll catabolites found in *Hydrangea aspera* D. Don ssp. sargentiana E. M. McClint autumnal leaves have been isolated from autumnal leaves of *Cercidiphyllum japonicum*, *Spinacia oleracea* and *Nicotiana rustica* [1, 2, 3]. Reverse phase high pressure liquid chromatography (RP-HPLC) methods have been used for the qualitative identification of the chlorophyll catabolites [1]. The hyphenated techniques provided the information on the *m/z* of the

[a] *University of Novi Sad, Technical Faculty "Mihajlo Pupin", Djure Djakovica bb, SRB-23000 Zrenjanin, Serbia, djapic@tfzr.uns.ac.rs*

chlorophyll catabolites and allowed the structural determination of the chlorophyll catabolites by their molecular mass [4]. The temperature plays an important role in the chromatographic separation of the chlorophyll catabolites. The temperature can influence the separation of compounds [5]. The role of temperature in RP-HPLC and RP liquid chromatography (RP-LC) has been used for the analysis of basic pharmaceuticals, water soluble vitamins, small peptides, etc [6, 7, 8]. The retention behavior of the *Hydrangea aspera* chlorophyll catabolites over the temperature range of 278K-318 K was investigated in order to collect the data on the effects of the temperature on the separation of the *Hydrangea aspera* chlorophyll catabolites on the $C_4$ and $C_8$ reverse phase (RP) analytical columns. The capacity factor was calculated for the chlorophyll catabolites present in autumnal leaves of *Hydrangea aspera*. The enthalpy and entropy changes of the *Hydrangea aspera* chlorophyll catabolites are reported.

## RESULTS AND DISCUSSION

### The *Hydrangea aspera* chlorophyll catabolites.

The LC – MS analysis of the *Hydrangea aspera* autumnal leaves dichloromethane and ethyl acetate extracts were subjected on the RP – $C_4$ and RP – $C_8$ analytical columns under the same acquisition parameters and elution solvent mixtures. The chromatograms obtained revealed the presence of the chlorophyll catabolites depicted in Fig.1.

The *Hydrangea aspera* autumnal leaves dichloromethane extract revealed the presence of nine chlorophyll catabolites when the separation was done on the RP – $C_4$ analytical column. The chlorophyll catabolite (**4**) with the m/z 677, eluted at 48.2 min. at the 298 K, assigned as 2 in Fig.2. Two isomers with the m/z 679, assigned 1 and 3 in the Fig.2, refers to the structure **3** in the Fig.1. Two isomers with the m/z 805 refers to the structure **6** in the Fig. 1 and where assigned 4 and 8 in Fig. 2. Two most abundant chlorophyll catabolites were with the m/z 807 (**5**) and were assigned in Fig. 2 with the numbers 5 and 6. The chlorophyll catabolites with the m/z 645 (**1**) and 643 (**2**) were also present and are assigned with the numbers 7 and 9 in the Fig. 2., respectively. The ESI-MS data of the chlorophyll catabolites numerated 3 (**3**) 5 (**5**), 7 (**1**) and 9 (**2**) in the Fig. 2 is depicted in Fig. 4. During the thermodynamical investigations chlorophyll catabolites numeration refers to the numbers they were assigned in Fig. 2.

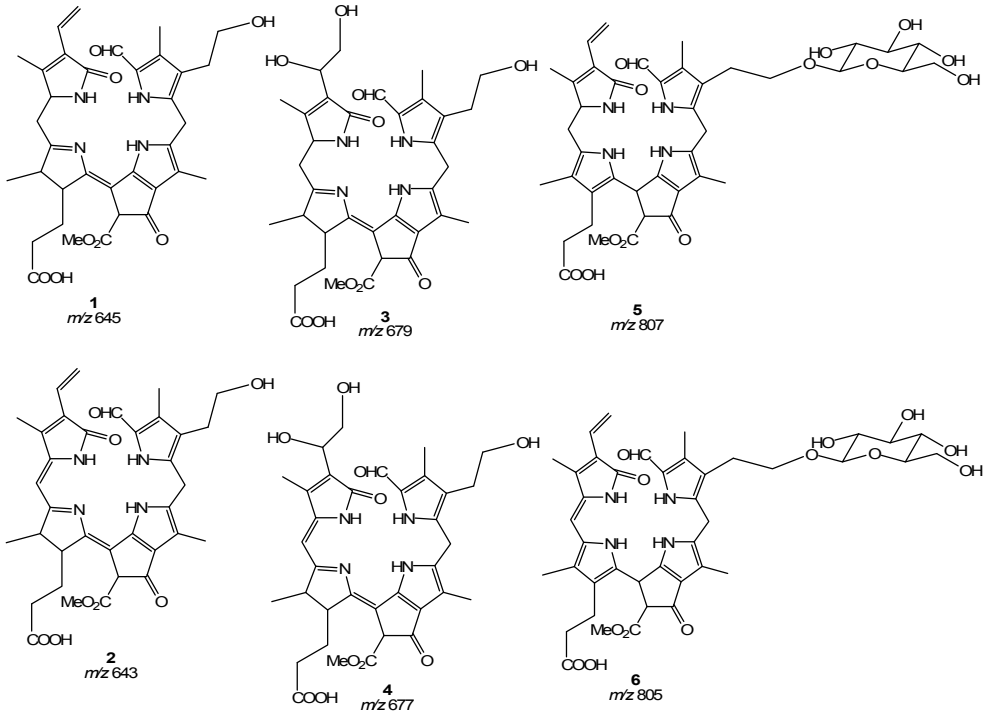


**Figure 1.** Chlorophyll catabolites present in *Hydrangea aspera* autumnal leaves dichloromethane and ethyl acetate extracts.

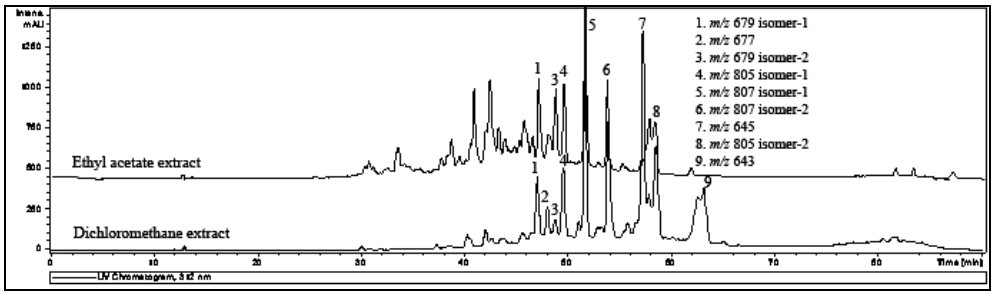


**Figure 2.** The chromatogram of *Hydrangea aspera* autumnal leaves' dichloromethane and ethyl acetate extract. The LC conditions: Column: Nucleosil 100-5 $C_4$ 4x250 mm. The mobile phase: 90% v/v water (0.1% TFA):methanol to 0% v/v water (0.1%TFA):methanol in 90 minutes. Flow rate: 0.2 ml $min^{-1}$. UV detection at λ=312. The oven temperature was 298 K.

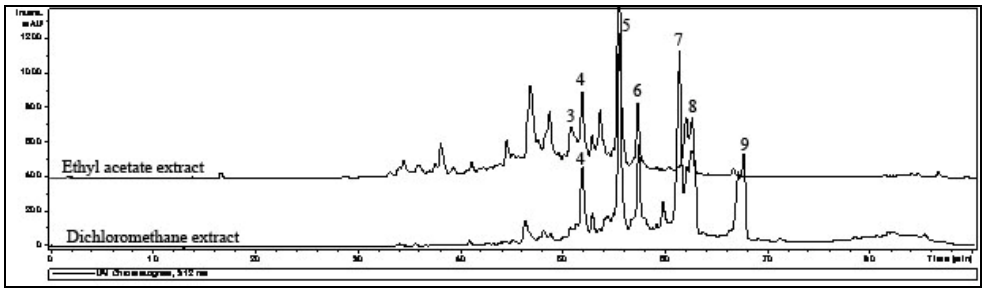


**Figure 3.** The chromatogram of *Hydrangea aspera* autumnal leaves' dichloromethane and ethyl acetate extract. The LC conditions: Column: Nucleosil 100-5 $C_8$ 4x250 mm. The mobile phase: 90% v/v water (0.1% TFA):methanol to 0% v/v water (0.1%TFA):methanol in 90 minutes. Flow rate: 0.2 ml min-1. UV detection at λ=312. The oven temperature was 298 K. The numeration of chlorophyll catabolites is as in the Fig. 2.

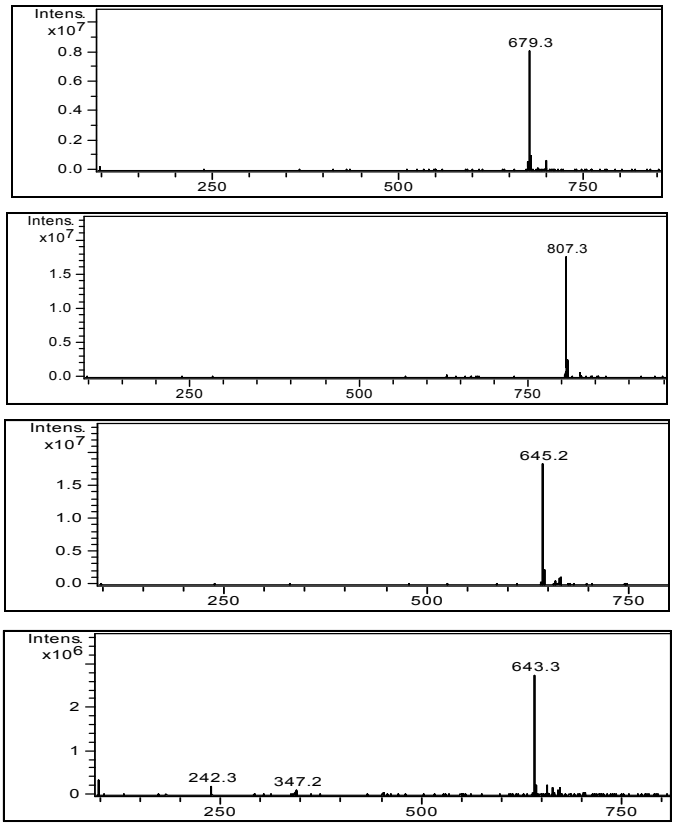


**Figure 4.** The ESI-MS of the chlorophyll catabolites numerated 3, 5, 7 and 9 in the Figure 2.

**The thermodynamic study on the separation of the *Hydrangea aspera* chlorophyll catabolites on the RP – $C_4$ and RP – $C_8$ analytical columns.** In case, when the separation was done on the RP – $C_4$ analytical column, the capacity factor k' showed a decrease, with a few exceptions at the temperature of 298K (Table 1). The capacity factor k' increases with the temperature in the case of all the *Hydrangea aspera* chlorophyll catabolites when the separation is done on the RP – $C_8$ analytical column (Table 2).

**Table 1.** Capacity factor of *Hydrangea aspera* chlorophyll catabolites on the RP-$C_4$ analytical column at different temperatures

| T [K] | 278 | 288 | 298 | 308 | 318 |
|---|---|---|---|---|---|
| *k'* of the m/z 679 isomer-1 | 0.44 | 0.41 | 0.41 | 0.39 | 0.38 |
| *k'* of the m/z 677 | 0.45 | 0.42 | 0.43 | 0.41 | 0.40 |
| *k'* of the m/z 679 isomer-2 | 0.46 | 0.43 | 0.44 | 0.42 | 0.41 |
| *k'* of the m/z 805 isomer-1 | 0.47 | 0.44 | 0.45 | 0.43 | 0.42 |
| *k'* of the m/z 807 isomer-1 | 0.49 | 0.46 | 0.47 | 0.45 | 0.43 |
| *k'* of the m/z 807 isomer-2 | 0.51 | 0.49 | 0.50 | 0.48 | 0.47 |
| *k'* of the m/z 645 | 0.54 | 0.52 | 0.53 | 0.52 | 0.51 |
| *k'* of the m/z 805 isomer-2 | 0.55 | 0.53 | 0.54 | 0.53 | 0.52 |
| *k'* of the m/z 643 | 0.59 | 0.57 | 0.58 | 0.57 | 0.57 |

*k'* – capacity factor.

**Table 2.** Capacity factor of *Hydrangea aspera* chlorophyll catabolites on the RP-$C_8$ analytical column at different temperatures

| T [K] | 278 | 288 | 298 | 308 | 318 |
|---|---|---|---|---|---|
| *k'* of the m/z 679 isomer-2 | 0.31 | 0.31 | 0.34 | 0.34 | 0.35 |
| *k'* of the m/z 805 isomer-1 | 0.31 | 0.31 | 0.34 | 0.34 | 0.36 |
| *k'* of the m/z 807 isomer-1 | 0.35 | 0.35 | 0.38 | 0.39 | 0.40 |
| *k'* of the m/z 807 isomer-2 | 0.37 | 0.38 | 0.41 | 0.41 | 0.43 |
| *k'* of the m/z 645 | 0.41 | 0.42 | 0.45 | 0.45 | 0.47 |
| *k'* of the m/z 805 isomer-2 | 0.42 | 0.43 | 0.46 | 0.46 | 0.48 |

*k'* – capacity factor.

The representative graphs of the retention factor logarithm versus the inverse temperature (van't Hoff plots) are shown in Fig. 5 and 6.

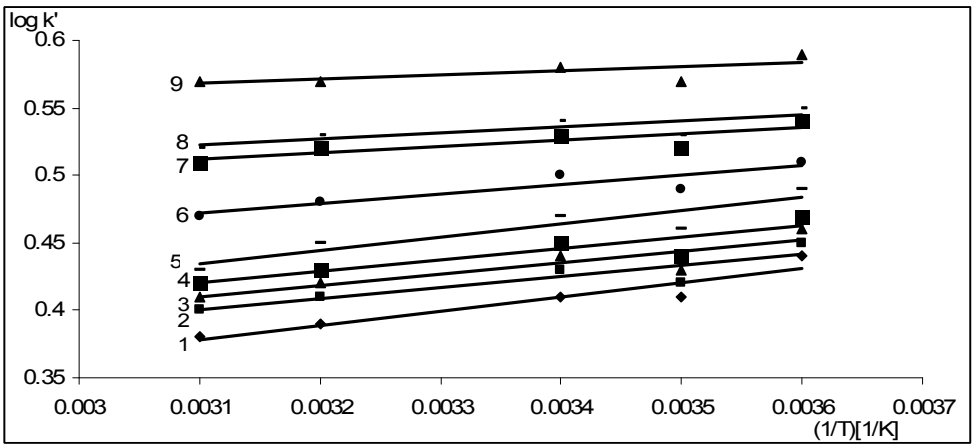


**Figure 5.** The graphs of the retention factor vs. inverse temperature used to calculate the change in molar enthalpy and entropy of the *Hydrangea aspera* chlorophyll catabolites, the: *m/z* 679 isomer-1 (graph 1), *m/z* 677 (graph 2), *m/z* 679 isomer-2 (graph 3), *m/z* 805 isomer-1 (graph 4), *m/z* 807 isomer-1 (graph 5), *m/z* 807 isomer-2 (graph 6), *m/z* 645 (graph 7), *m/z* 805 isomer-2 (graph 8) and *m/z* 643 (graph 9) on the RP-$C_4$ analytical column.

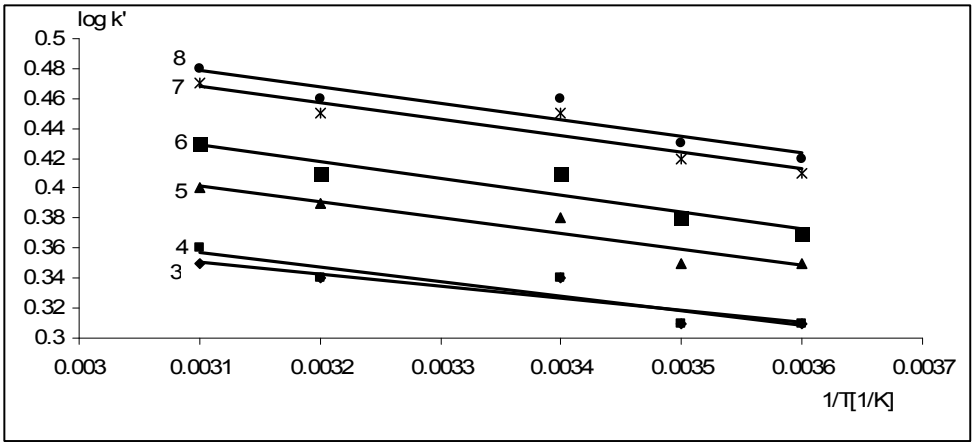


**Figure 6.** The graphs of the retention factor vs. inverse temperature used to calculate the change in molar enthalpy and entropy of the *Hydrangea aspera* chlorophyll catabolites, the: *m/z* 679 isomer-2 (graph 3), *m/z* 805 isomer-1 (graph 4), *m/z* 807 isomer-1 (graph 5), *m/z* 807 isomer-2 (graph 6), the *m/z* 645 (graph 7) and the *m/z* 805 isomer-2 (graph 8) on the RP-$C_8$ analytical column.

The obtained graphs were approximated to be linear although the square of the correlation coefficient were variable in case when the separation was done on the RP-$C_4$ analytical column, while when the separation was done on the RP-$C_8$, the square of the correlation coefficient were in the range of $R^2$=0.81 – 0.91 (Table 3 and 4). The linear graph would have indicated that the change in molar enthalpy is constant and that there is no significant change in retention mechanism over the temperature range of 278 – 318 K. The slopes are negative for the separations on the RP – $C_8$ analytical column indicating a positive change in molar enthalpy suggesting that the transfer from the mobile to the stationary phase is enthalpically unfavorable. The changes in molar enthalpies were calculated from the slopes in Fig. 5 and 6, according to the equation (2) and are depicted in Tables 3 and 4. The phase ratio of the column (Φ) was assumed to be a constant.

**Table 3.** Capacity factor of *Hydrangea aspera* chlorophyll catabolites on the RP-$C_4$ analytical column at different temperatures

| | ΔH [J mol$^{-1}$] | ΔS [J mol$^{-1}$] | $R^2$ |
|---|---|---|---|
| *k'* of the m/z 679 isomer-1 | -2.02 | 0.97 | 0.91 |
| *k'* of the m/z 677 | -1.60 | 2.69 | 0.81 |
| *k'* of the m/z 679 isomer-2 | -1.60 | 2.88 | 0.81 |
| *k'* of the m/z 805 isomer-1 | -1.60 | 3.07 | 0.81 |
| *k'* of the m/z 807 isomer-1 | -1.89 | 2.45 | 0.84 |
| *k'* of the m/z 807 isomer-2 | -1.33 | 4.88 | 0.84 |
| *k'* of the m/z 645 | -0.87 | 7.11 | 0.68 |
| *k'* of the m/z 805 isomer-2 | -0.87 | 7.30 | 0.68 |
| *k'* of the m/z 643 | -0.58 | 9.07 | 0.49 |

ΔH – molar enthalpy, ΔS – molar entropy, $R^2$ – the square of the correlation coefficient.

**Table 4.** Capacity factor of *Hydrangea aspera* chlorophyll catabolites on the RP-$C_8$ analytical column at different temperatures

| | ΔH [kJ mol$^{-1}$] | ΔS [J mol$^{-1}$] | $R^2$ |
|---|---|---|---|
| *k'* of the m/z 679 isomer-2 | 1.56 | 11.54 | 0.81 |
| *k'* of the m/z 805 isomer-1 | 1.85 | 12.55 | 0.85 |
| *k'* of the m/z 807 isomer-1 | 2.02 | 13.95 | 0.91 |
| *k'* of the m/z 807 isomer-2 | 2.11 | 14.75 | 0.87 |
| *k'* of the m/z 645 | 2.11 | 15.51 | 0.87 |
| *k'* of the m/z 805 isomer-2 | 2.11 | 15.70 | 0.87 |

ΔH – molar enthalpy, ΔS – molar entropy, $R^2$ – the square of the correlation coefficient.

When molar enthalpies are compared during the separation on the RP – $C_4$ analytical column there is an increase in molar entropy from the first *Hydrangea aspera* chlorophyll catabolite, with the *m/z* 679 isomer-1 to the last eluting *Hydrangea aspera* chlorophyll catabolite, with the *m/z* 643. The only exception was the *Hydrangea aspera* chlorophyll catabolite, with the *m/z* 807 isomer-1.

## CONCLUSIONS

The extraction of *Hydrangea aspera* chlorophyll catabolites from the autumnal leaves' methanol extract with dichloromethane and ethyl acetate differs slightly. The identification of *Hydrangea aspera* chlorophyll catabolites on the RP –$C_4$ column reveals the presence of nine chlorophyll catabolites, while the separation on the RP –$C_8$ reveals the presence of few less chlorophyll catabolites. The thermodynamic investigations indicated that the retention behaviour of *Hydrangea aspera* chlorophyll catabolites on RP – $C_4$ and RP – $C_8$ analytical columns were slightly driven by the enthalpy difference. The van't Hoff curves obtained were approximated to be linear. When the investigations were done on the RP – $C_4$ the deviation from the linear approximation was great in case of the *Hydrangea aspera* chlorophyll catabolites with the *m/z* 643. The next investigations in reversed – phase liquid chromatography of chlorophyll catabolites are desirable. The search for the optimal mobile phases, modifiers and buffers is necessary in order to find the best separation conditions for the separation of the chlorophyll catabolites.

## EXPERIMENTAL SECTION

*Hydrangea aspera* D. Don ssp. Sargentiana E. M. McClint autumnal leaves (15g dry weight, 20g "fresh" weight) were chilled with liquid nitrogen, grinded and homogenized in a blender with 0.2 $dm^3$ 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. The obtained volume was divided in two parts. From one part *Hydrangea aspera* chlorophyll catabolites were extracted with dichloromethane from the aqueous phase. Evaporation of dichloromethane ($t<40^oC$) yielded 12.53 mg. From the other part *Hydrangea aspera* chlorophyll catabolites were extracted with ethyl acetate from the aqueous phase. Evaporation of ethyl acetate ($t<40^oC$) yielded 10.84 mg. The extracts obtained were dissolved in methanol and subjected to the 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/ESI – MS analysis were performed on Waters 2695 Separations Module (Milford, MA, USA) coupled to a Waters 2996 PDA UV-Vis detector and connected to Bruker Daltonics esquire 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 ESI experiments. The ESI detection was done in positive mode. The capillary voltage in a ramp ranged from 4.5 to 1.5 kV. Data were acquired by HyStarTM and processed by Bruker Daltonics Data Analysis running under Windows NTTM (Microsoft, Redmond, USA). The LC separations were carried on the reverse phase (RP) EC 250x4 mm Nucleosil® 100-5 $C_8$ column together with RP CC 8x4 mm Nucleosil® 100-5 $C_8$ precolumn and the 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 10μL via autosampler injection and in every sample 10 μL of uracil (0.01 mg $mL^{-1}$) was dissolved. For the thermodynamical investigations the temperature of the column oven was in a range from 278 K to 318 K. The starting measurement was done at oven temperature of 278 K with the subsequent increase of temperature by 10 K. 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 with a flow rate of 0.2 mL $min^{-1}$. After each separation the column was reequilibrated linearly from 100 % methanol to 90% water (0.1% TFA):10% methanol in 10 minutes and additional 5 minutes at 90% water (0.1% TFA):10% methanol. Data were acquired by HyStarTM and processed by Bruker Daltonics Data Analysis running under Windows NT™ (Microsoft, Redmond, USA).

The following formulas were used for the calculation of the capacity factor and the van't Hoff isotherm [9].
The capacity factor (k') was calculated with the equation (1):

$$k' = \frac{t_R - t_0}{t_0} \quad (1)$$

where the $t_R$ is the retention time of one of the *Hydrangea aspera* chlorophyll catabolite and $t_0$ is the retention time of the unretained compound (uracil).
The van't Hoff equation [10]:

$$\log k' = (-\frac{\Delta H}{2.3\,RT}) + (\frac{\Delta S}{2.3R}) + \log \Phi \quad (2)$$

where ΔH is enthalpy, ΔS is entropy, T is the absolute temperature, R is the universal gas constant and the Φ is the phase ratio of the system. In the van't Hoff plot the log k' versus 1/T is usually a linear curve with the slope of – ΔH/2.3R and an intercept of ΔS/2.3R+log Φ. The value of Φ was assumed to remain constant over the temperature range studied, so that the general trends in ΔS could be analyzed [6].

The values obtained during the experimental measurements represent the means of the triplicate measurements (n=3) ± SD.

## REFERENCES

1. M. Oberhuber, J. Berghold, K. Breuker, S. Hoertensteiner, B. Kraeutler, *Proceedings of the National Academy of Sciences USA*, **2003**, 100, 6910.
2. M. Oberhuber, J. Berghold, W. Muehlecker, S. Hoertensteiner, B. Kraeutler, *Helvetica Chimica Acta*, **2001**, 84, 2615.
3. J. Berghold, C. Eichmueller, S. Hoertensteiner, B. Kraeutler, *Chemistry & Biodiversity*, **2004**, 1, 657.
4. T. Mueller, S. Oradu, D. R. Ifa, R.G. Cooks, B. Kraeutler, *Analytical Chemistry*, **2011**, 83, 5754.
5. C.B. Castells, P. W. Carr, *Chromatographia*, **2000**, 52, 535.
6. R.J.M. Vervoort, E. Ruyter, A.J.J. Debets, H.A. Claessens, C.A. Cramers, G.J. de Jong, *Journal of Chromatography A*, **2002**, 964, 67.
7. V.A. Chirkin, S.I. Karpov, V.F. Selemenev, *Russian Journal of Physical Chemistry A*, **2012**, 86, 1903.
8. C.-W. Tsai, C.-I. Liu, Y.-C. Chan, H.-H. Tsai, R.-C. Ruaan, *Chromatographia*, **2010**, 114, 11620.
9. R.E. Ardrey, "Liquid chromatography – mass spectrometry: An Introduction", Wiley, England, **2003**, chapter 2.
10. A. Tchapla, S. Heron, H. Colin, G. Guichon, *Analytical Chemistry*, **1988**, 60, 1443.