

# Using of liquid chromatography coupled with diode array detector for determination of naphthoquinones in plants and for investigation of influence of pH of cultivation medium on content of plumbagin in *Dionaea muscipula*

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## Abstract

The interest of many investigators in naphthoquinones is due to their broad-range of biological actions from phytotoxic to fungicidal. The main aim of this work was to investigate the influence of different pH values of cultivation medium on naphthoquinone content in *Dionaea muscipula*. For this purpose, we optimized the simultaneous analysis of the most commonly occurring naphthoquinones (1,4-naphthoquinone, lawsone, juglone and plumbagin) by high performance liquid chromatography coupled with diode array detector (HPLC-DAD). The most suitable chromatographic conditions were as follows: mobile phase: 0.1 mol l<sup>-1</sup> acetic acid:methanol in ratio of 33:67 (% v/v), flow rate: 0.75 ml min<sup>-1</sup> and temperature: 42 °C. Moreover, we looked for the most suitable technique for preparation of plant samples (*D. muscipula*, *Juglans regia*, *Paulownia tomentosa*, *Impatiens glandulifera*, *Impatiens parviflora*, *Drosera rotundifolia*, *Drosera spathulata* and *Drosera capensis*) due to their consequent analysis by HPLC-DAD. It clearly follows from the results obtained that sonication were the most suitable technique for preparation of *J. regia* plants. We also checked the recoveries of the determined naphthoquinones, which were from 96 to 104%. Finally, we investigated the changes in content of plumbagin in *D. muscipula* plants according to different pH of cultivation medium. The content increased with increasing pH up to 5 and, then, changed gradually. The lower content of plumbagin at lower pH values was of interest to us. Therefore, we determined the content of this naphthoquinone in the cultivation medium, what has not been studied before. We discovered that the lower tissue content of plumbagin was due to secretion of this naphthoquinone into the cultivation medium.

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## 1. Introduction

Interaction of species in an ecosystem is known to be determined by many factors, including the ability of microorganisms or plants to excrete substances that affect the development of other species. Such substances called allelopathic metabolites cover a broad-range of metabolites including both simple (organic acids, sugars, etc.) and complex (hormones, secondary metabolites, etc.) compounds inhibiting and/or stimulating metabolic processes [1].

Naphthoquinones are one of the secondary metabolic groups widespread in nature, where they mostly appear as chromatic pigments. They have been found in higher plants such as *Plumbaginaceae*, *Juglandaceae*, etc. [2–5], fungi (*Marasmius gramineus* and *Verticillium dahliae*) [1] and microorganisms (*Streptomyces* and *Fusarium*) [6]. Plant extracts containing naphthoquinones have been used for a long time in traditional medicines of number of nations for cancer and rheumatoid arthritis treatment, particularly extracts from *Plumbago europaea* for mitigation of toothache, extracts from *Plumbago zeylanica* for treatment of diarrhoea, skin diseases and digestion malfunction [7,8].

The interest of many investigators in this class of compounds is due to their broad-range of biological activities: phytotoxic

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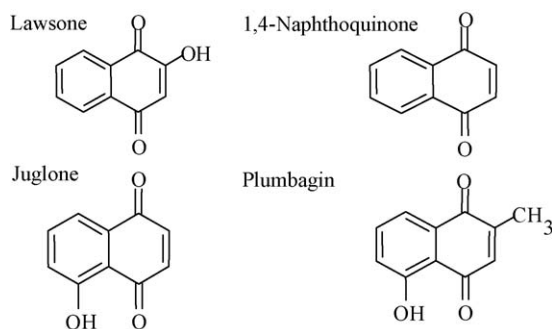


Fig. 1. Chemical structures of 1,4-naphthoquinone, lawsone, juglone and plumbagin.

[9–11], insecticidal [1], antibacterial [12–14] and fungicidal [13,14]. Besides that they have also cytostatic [13] and anti-carcinogenic [1] properties. Their cytostatic and antimicrobial activities emerge due to their ability to act as potent inhibitors of electron transport [15], as uncouplers of oxidative phosphorylation [16], as intercalating agents in the DNA double helix, as bioreductive alkylating agents of biomolecules, and as producers of reactive oxygen radicals by redox cycling under aerobic conditions [16,17]. An anticancer effect of naphthoquinones stimulates an interest in determination and characterization of single derivatives of 1,2- and 1,4-quinones in biological samples. High performance liquid chromatography with UV detection and electrochemical techniques are the most commonly used methods for these purposes [18–21]. In addition de Paiva et al. [22] used mass spectrometry as a tool for the study of plumbagin. The main aim of this work was to investigate the influence of different pH values of cultivation medium on naphthoquinone content in Venus's flytrap (*D. muscipula*). For these purposes it was necessary to optimize the simultaneous analysis of the most commonly occurring naphthoquinones (1,4-naphthoquinone, lawsone, juglone and plumbagin; Fig. 1) by high performance liquid chromatography coupled with a diode array detector (HPLC-DAD). The optimized technique was consequently used for detection and quantification of naphthoquinones in plant materials (*D. muscipula*, *Juglans regia*, *Paulownia tomentosa*, *Impatiens glandulifera*, *Impatiens parviflora*, *Drosera rotundifolia*, *Drosera spathulata* and *Drosera capensis*).

## 2. Materials and methods

### 2.1. Chemicals

Naphthoquinones (1,4-naphthoquinone, lawsone, juglone and plumbagin) were purchased from Sigma Aldrich Chemical Corp. (St. Louis, USA). Methanol for HPLC and other analytical reagents of ACS purity were also purchased from Sigma Aldrich. Solutions were prepared using deionised ACS water (Sigma). The stock standard solutions of naphthoquinones at (100  $\mu\text{g ml}^{-1}$ ) were prepared in ACS methanol and stored in the dark at 4 °C. The working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through a 0.45  $\mu\text{m}$  Teflon membrane filter (MetaChem, Torrance, USA) prior to HPLC separations. The pH value was

measured using WTW inoLab Level 3 (Weilheim, Germany) controlled by a personal computer program (MultiLab Pilot; Weilheim, Germany).

### 2.2. Plant materials

*D. muscipula* Ell. plants were originally established at the Department of Natural Drugs of the University of Veterinary and Pharmaceutical Sciences in Brno, and *D. rotundifolia* L., *D. spathulata* Labill. and *D. capensis* L. at the Faculty of Science of Masaryk University in Brno, Czech Republic. The whole plants were cultivated on 20 ml of Murashige Skoog Medium (MS) with addition of sucrose (30 g/l) and activated carbon (1.5 g/l) in Erlenmeyer flasks (250 ml); the total volume of cultivation medium was 50 ml. The plants were cultivated in cultivation boxes (laminar box Aura Mini, BioTech Instruments, Prague, Czech Republic) for 1 month, 14 h long daylight per day (maximal light intensity was about 100  $\mu\text{Em}^{-2} \text{s}^{-1}$ ) at a temperature  $26 \pm 0.5$  °C and humidity  $60 \pm 5\%$ . Sub-cultivation of stock cultures was done at 2-month intervals. pH values of cultivation medium (*D. muscipula*) was adjusted by addition of 1 M NaOH and/or 1 M HCl.

*P. tomentosa* (Thunb.) Sieb. & Zucc. ex Steud., *J. regia* L., *I. glandulifera* Royle and *I. parviflora* DC. were grown in a garden placed at the University of Veterinary and Pharmaceutical Sciences, Brno.

### 2.3. HPLC analysis

An HP 1100 liquid chromatographic system (Hewlett Packard, Waldbronn, Germany) was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an auto sampler (G1313A), a column thermostat (G1316A), and a UV–vis diode array detector (model G1315B) working at 190–690 nm. ChemStation software (Rev. A 08.01) was used to control the entire liquid chromatographic system. Spectra were registered in the range of 190–400 nm (SBW 100 nm). The wavelength used for simultaneous determination of the naphthoquinones of interest was 260 nm. Naphthoquinones were separated on a reversed-phase Zorbax C18-AAA chromatographic column (150 mm  $\times$  4.6 mm, 3.5  $\mu\text{m}$  particle size, Agilent Technologies, USA) in an isocratic mode. Auto sampler injection was 5  $\mu\text{l}$ . Internal standard was 1,4-quinone. For other chromatographic conditions such as composition of mobile phase, flow rate of mobile phase, see Section 3.

### 2.4. Sample preparation

After weighing, leaves of *J. regia* were immediately chopped and extracted using hexane, methanol or acetonitrile. Accelerated solvent extraction (ASE) was performed using a PSE extractor (Applied Separations, USA) [23,24]. Briefly, the sample (*J. regia*; 0.10 g  $\pm$  5 mg) was wrapped with filter paper (small envelope-like format) and sprinkled with SPE-ed<sup>TM</sup> matrix. 1,4-quinone was used as an internal standard and was pipetted onto the top of the sample. The mixture was placed into a 10 ml stainless steel extraction cell and extracted under controlled

conditions with a pre-heating period (5 min), the solvent hexane; temperature 40 °C, pressure 150 bar; two extraction cycles (5 min), and 90 s using pressurized nitrogen. The final extracts were collected in 30 ml glass vials with Teflon coated rubber caps and evaporated to dryness in a rotary vacuum evaporator (IKA RV 05-ST) with an HB 4 water bath (both, IKA-Werke GmbH and Co., Staufen, KG, Germany). The residue was reconstituted in 1 ml methanol and filtered through a 0.45 µm Teflon membrane filter (MetaChem, Torrance, CA, USA) prior to injection into the HPLC system.

Plant samples (*D. muscipula*, *Drosera sp.*, *Impatiens sp.*, *P. tomentosa* and *J. regia*) were lyophilized at –51 °C for 48 h (CHRIST-Alpha 1-2). Then the lyophilized samples were homogenized by an Ika A11 basic grinder (IKA Werke GmbH and Co., Staufen, KG, Germany). The homogenized samples (0.02–0.10 g) were dissolved in 5 ml of 99.999% methanol and sonicated at the laboratory temperature for 30 min using a K5 Sonicator (Czech Republic) at 150 W, 38 kHz. The samples were filtered through a 0.45 µm Teflon membrane filter (MetaChem) prior to injection into the HPLC system.

### 2.5. Accuracy, precision and recovery

Accuracy, precision and recovery of naphthoquinones were evaluated with homogenates of the analyzed plant tissues spiked with standards. Before extraction was carried out, 100 µl naphthoquinone standards (concentrations varying from 10 to 2500 µg g<sup>-1</sup>), 100 µl water and 100 µl 1,2-quinone were added to the plant tissue sample. Precision (coefficient of

variation; % CV) of intra-day assay was performed using six homogenates. Inter-day precision was determined by analyzing six homogenates over a 5-day period. Homogenates were assayed blindly and naphthoquinone concentrations were derived from the calibration curves. Accuracy was evaluated by comparing the estimated concentration with the known concentrations of naphthoquinones. Calculation of accuracy (% Bias), precision (% CV) and recovery was expressed according to refs. [25–27].

### 2.6. Statistical analysis

STATGRAPHICS® (Statistical Graphics Corp.®, USA) was used for statistical analyses. Results are expressed as mean ± S.D. unless noted otherwise. Value of  $p < 0.05$  was considered significant.

## 3. Results and discussion

The development of new analytical techniques used for a determination of biologically active compounds is one of the most important tasks of analytical chemistry, biochemistry and pharmacy [24,27–35]. Here we wished to study the content of naphthoquinones (1,4-naphthoquinone, lawsone, juglone and plumbagin; Fig. 1) in different plant species by HPLC-DAD. Therefore, primarily it was necessary to optimize their simultaneous analysis. The HPLC-DAD chromatographic determination of naphthoquinones has been described by several authors [18,36,37]. Nevertheless, here we used a new technique for the

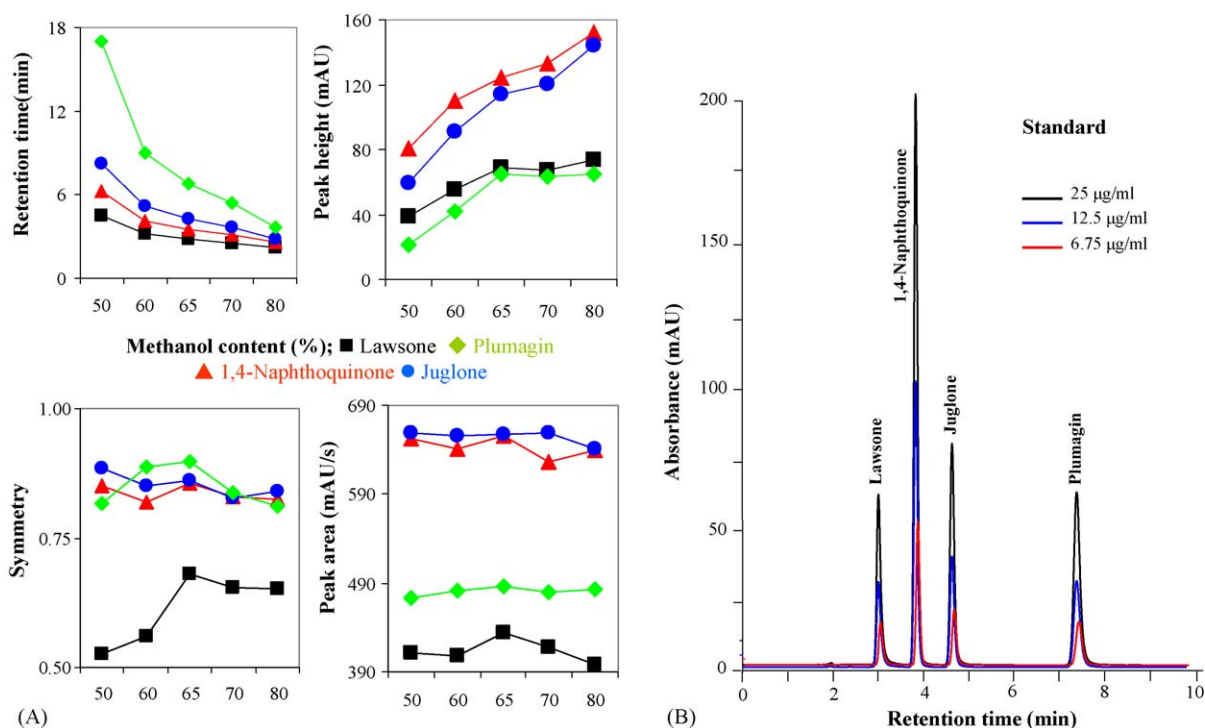


Fig. 2. An influence of different methanol content in a mobile phase on retention time, peak height, symmetry and peak area of the studied naphthoquinones (A). HPLC-DAD chromatograms of 1,4-naphthoquinone, lawsone, juglone and plumbagin at three different concentrations of 6.75, 12.5 and 25 µg ml<sup>-1</sup> (B). HPLC-DAD conditions were as follows: mobile phase: 0.1 mol l<sup>-1</sup> acetic acid:methanol in ratio of 33:67 (% , v/v) except (A); flow rate: 0.75 ml min<sup>-1</sup>; temperature: 42 °C. Chromatograms were registered at 260 nm. For other details see Section 2.

Table 1  
HPLC-DAD characteristics of analysed naphthoquinones ( $n = 5$ )

Naphthoquinone	$t_R$ (min)	Equation <sup>a</sup>	$R^2$	LOD (ng ml <sup>-1</sup> ) <sup>b</sup>	LOQ (ng ml <sup>-1</sup> ) <sup>b</sup>	R.S.D. (%) <sup>b</sup>
1,4-Naphthoquinone	3.78	$y = 7.9563x - 0.7992^b$ $y = 45.988x - 4.2282^c$	0.9997 <sup>b</sup> 0.9999 <sup>c</sup>	8	26.6	1.91
Juglone	4.67	$y = 3.1169x - 0.5831^b$ $y = 20.887x - 3.2387^c$	0.9998 <sup>b</sup> 0.9997 <sup>c</sup>	21	69.9	2.25
Lawsone	3.05	$y = 2.3858x - 0.6076^b$ $y = 15.716x - 2.4979^c$	0.9998 <sup>b</sup> 0.9993 <sup>c</sup>	28	94.0	2.86
Plumbagin	7.49	$y = 2.4026x - 0.2810^b$ $y = 24.814x - 4.2983^c$	0.9997 <sup>b</sup> 0.9998 <sup>c</sup>	27	88.5	1.11

<sup>a</sup> The concentration range was from 0.1 to 35  $\mu\text{g ml}^{-1}$ .

<sup>b</sup> The equation was derived from the dependence of the peak height on the naphthoquinone concentration.

<sup>c</sup> The equation was derived from the dependence of the peak area on the naphthoquinone concentration.

evaluation of chromatographic parameters according to Potesil et al. [27]. We found that changing the ratio of aqueous and organic part of the mobile phase markedly influenced separation and detection of the studied naphthoquinones. To be specific, we changed the ratio of methanol and 0.1 mol l<sup>-1</sup> acetic acid (50, 60, 65, 70 and 80% of methanol; v/v) and observed changes in retention time, peak height, symmetry and peak area (Fig. 2A). The peak heights increased and peak area did not change with increasing methanol content. Moreover the retention times of naphthoquinones decreased, but the resolution of lawsone and 1,4-naphthoquinone signals was poor at a content of methanol of more than 70% (Fig. 2A). It is evident that the most suitable content of methanol lies between 60 and 70%. Therefore, we thoroughly studied this interval (not shown). On the basis of the obtained results we selected the ratio of acetic acid:methanol of 33:67 (% v/v) as the most suitable for the simultaneous determination of naphthoquinones. We also investigated the influence of other chromatographic conditions such as flow rate of the mobile phase and temperature on the simultaneous determination of naphthoquinones and found out that the most suitable conditions were as followed: mobile phase: 0.1 mol l<sup>-1</sup> acetic acid:methanol in ratio of 33:67 (% v/v); flow rate: 0.75 ml min<sup>-1</sup>; temperature: 42 °C. We obtained well developed, separated, reproducible and symmetrical signals of the studied naphthoquinones using the mentioned conditions (Fig. 2B). In addition the dependence of an absorbance on naphthoquinone concentration were strictly linear in the stud-

ied concentration interval (0.1–35  $\mu\text{g ml}^{-1}$ , Table 1). Relative standard deviations (R.S.D.) were about 3% ( $n = 5$ ). The calibration curve equations, LODs and LOQs are shown in Table 1.

### 3.1. Extraction of naphthoquinones

As soon as we optimized the HPLC-DAD determination of naphthoquinones, we applied this technique for the determination of the naphthoquinones in plants. Therefore, it was necessary to select the most effective technique for a preparation of a real sample. We have found that a number of authors have extracted the naphthoquinones by different organic solvents using different extraction conditions, but authors, which have compared the effectiveness of solvents, have not been found. Primarily we tested the influence of hexane, methanol or acetonitrile on amounts of juglone extracted from leaves of walnut (*J. regia*), which contain higher amounts of this naphthoquinone [5,19]. When we analyzed the extracts, we observed the signal of juglone at a retention time of 5 min (not shown). The highest yield of juglone was obtained when methanol was used as an extraction solvent and the lowest when we used hexane (Fig. 3a). In addition we tested the influence of temperature on the yield of juglone using accelerated solvent extraction (ASE). We found that the yield of juglone markedly decreased with increasing temperature (more than 60% at 60 °C, Fig. 3b). Moreover, we tried to extract 1,4-naphthoquinone, but free 1,4-naphthoquinone does not occur in plant samples. The extraction

Table 2  
Recovery of naphthoquinone compounds from *Dionaea muscipula*, *Drosera rotundifolia*, *Juglans Regia*, *Impatiens glandulifera* and *Paulownia tomentosa* plants ( $n = 3$ )

Plant species	Naphthoquinone	Homogenate ( $\mu\text{g g}^{-1}$ ) <sup>a,b,c</sup>	Spiking naphthoquinone ( $\mu\text{g g}^{-1}$ ) <sup>a</sup>	Homogenate + spiked naphthoquinone ( $\mu\text{g g}^{-1}$ ) <sup>a</sup>	Recovery (%)
<i>Dionaea muscipula</i>	Plumbagin	8267 ± 200 (2.4)	2508 ± 86.9 (3.5)	10379 ± 529 (5.1)	96
<i>Drosera rotundifolia</i>	Plumbagin	22.3 ± 0.7 (3.1)	9.9 ± 0.4 (4.0)	31.7 ± 1.1 (3.5)	98
<i>Juglans regia</i>	Juglone	4980 ± 160 (3.2)	1098 ± 48.9 (4.5)	6325 ± 258 (4.1)	104
<i>Impatiens glandulifera</i>	Lawsone	539 ± 14 (2.6)	108 ± 3.9 (3.6)	665 ± 25 (3.8)	103
	Plumbagin	41.2 ± 1.4 (3.4)	10.4 ± 0.2 (1.9)	50.4 ± 1.6 (3.2)	98
<i>Paulownia tomentosa</i>	Plumbagin	29.1 ± 0.9 (3.1)	9.9 ± 0.2 (2.0)	40.1 ± 1.0 (2.5)	100

<sup>a</sup> Amounts of the naphthoquinones per gram of dry weight.

<sup>b</sup> Results expressed as a mean ± S.D. (CV %).

<sup>c</sup> Internal standard (quinone) was added.

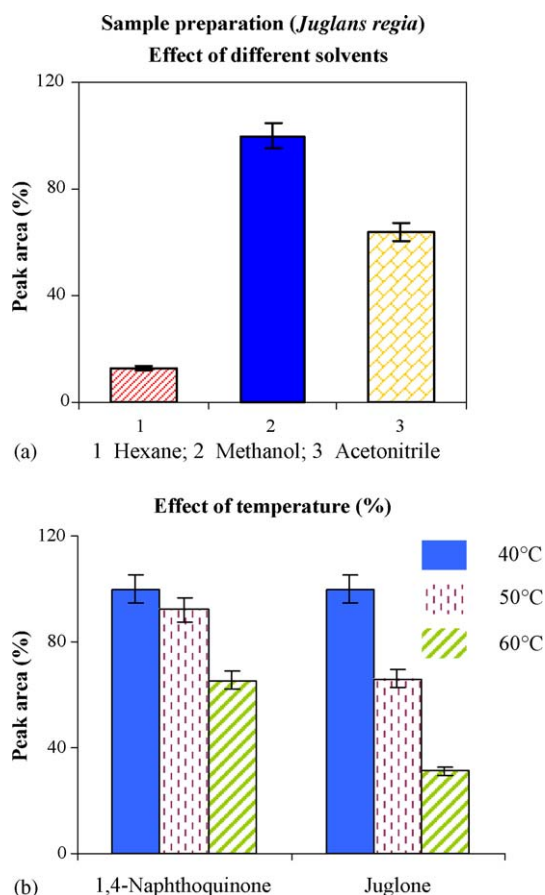


Fig. 3. Sample preparation (leaves of *Juglans regia*). An effect of different solvents (a) and temperature (b) on yield of juglone (a and b) and 1,4-naphthoquinone (b). Peak area of 111 mAU/s (a) and 223/246 mAU/s (juglone/1,4-naphthoquinone, (b) corresponds to the 100%. For other details see Sections 2 and 3.

of this naphthoquinone depends on the preparation of a sample using ASE. Using higher temperatures and pressures in the extraction procedure caused release of the 1,4-naphthoquinone from juglone. It clearly follows from the obtained results that ASE is not suitable for the preparation of plant tissues due to decomposition of studied naphthoquinone derivatives. Therefore, we prepared the walnut leaves by sonication (for other details see Section 2). We obtained higher amounts of juglone using sonication in comparison with the ASE technique and, moreover, did not detect any 1,4-naphthoquinone. Sonication appears to be more compatible with naphthoquinones and uses lower volumes of extraction solvents. That is why sonication was the most suitable method for extraction of naphthoquinones from plant tissues. In addition we determined the extraction efficiency of the studied naphthoquinones from *D. muscipula*, *J. regia*, *P. tomentosa*, *I. glandulifera*, *I. parviflora*, *D. rotundifolia*, *D. spathulata* and *D. capensis* tissues. To be specific, recovery was checked for the compounds of interest by addition of known amounts of the determined naphthoquinone working standards to homogenates (Table 2). Recoveries of the determined naphthoquinones ranged from 96 to 104% (Table 2). Reproducibility of the procedure was tested by analyzing representative samples in six replicates over 5 days (Table 3). Good precision was obtained for all naphthoquinones in plant samples with % CVs ranging from 2.4 to 4.9% in the intra-assay. The inter-assay % CVs ranged from 3.1 to 6.8%; overall recoveries were from 95 to 105% ( $n = 30$ ) and accuracy (% Bias) was about  $\pm 5\%$ .

### 3.2. Analysis of plant samples

We applied the optimized HPLC-DAD technique for analysis of plant tissue samples (*D. muscipula*, *J. regia*, *P. tomentosa*, *I. glandulifera*, *I. parviflora*, *D. rotundifolia*, *D. spathulata* and *D. capensis*). The obtained chromatograms of analysis of extracts from *D. muscipula*, *D. rotundifolia*, *I. glandulifera* and *P. tomen-*

Table 3  
Precision and recovery of naphthoquinones for *Dionaea muscipula*, *Drosera rotundifolia*, *Juglans Regia*, *Impatiens glandulifera* and *Paulownia tomentosa* plants

Plant specie		Naphthoquinone	Homogenate ( $\mu\text{g g}^{-1}$ ) <sup>a,b,c</sup>	Spiking naphthoquinone ( $\mu\text{g g}^{-1}$ ) <sup>a</sup>	Homogenate + spiked naphthoquinone ( $\mu\text{g g}^{-1}$ ) <sup>a</sup>	Recovery (%)
<i>Dionaea muscipula</i>	Intra-day ( $n = 6$ )	Plumbagin	8245 $\pm$ 196 (2.4)	2478 $\pm$ 96 (3.9)	10496 $\pm$ 515 (4.9)	98
	Inter-day ( $n = 30$ )	Plumbagin	8185 $\pm$ 257 (3.1)	2 611 $\pm$ 100 (3.9)	10254 $\pm$ 635 (6.2)	95
<i>Drosera rotundifolia</i>	Intra-day ( $n = 6$ )	Plumbagin	22.1 $\pm$ 0.6 (2.7)	10.0 $\pm$ 0.4 (4.0)	31.9 $\pm$ 0.7 (2.2)	99
	Inter-day ( $n = 30$ )	Plumbagin	23.1 $\pm$ 0.9 (3.9)	10.2 $\pm$ 0.5 (4.9)	35.1 $\pm$ 1.1 (3.5)	105
<i>Juglans regia</i>	Intra-day ( $n = 6$ )	Juglone	5025 $\pm$ 135 (2.7)	1048 $\pm$ 34 (3.2)	6115 $\pm$ 189 (3.1)	101
	Inter-day ( $n = 30$ )	Juglone	5145 $\pm$ 197 (3.8)	1057 $\pm$ 59 (5.6)	6446 $\pm$ 324 (5.0)	104
<i>Impatiens glandulifera</i>	Intra-day ( $n = 6$ )	Lawson	518 $\pm$ 15 (2.9)	111 $\pm$ 4.0 (3.6)	642 $\pm$ 28 (4.4)	102
	Inter-day ( $n = 30$ )	Plumbagin	41.4 $\pm$ 1.6 (3.9)	10.6 $\pm$ 0.4 (3.8)	50.2 $\pm$ 1.9 (3.8)	97
	Intra-day ( $n = 6$ )	Lawson	546 $\pm$ 25 (4.6)	115 $\pm$ 5.0 (4.3)	689 $\pm$ 34 (4.9)	104
	Inter-day ( $n = 30$ )	Plumbagin	42.4 $\pm$ 1.8 (4.2)	10.6 $\pm$ 0.6 (5.7)	54.1 $\pm$ 2.7 (5.0)	102
<i>Paulownia tomentosa</i>	Intra-day ( $n = 6$ )	Plumbagin	29.5 $\pm$ 1.1 (3.7)	9.9 $\pm$ 0.3 (3.0)	39.8 $\pm$ 1.4 (3.5)	101
	Inter-day ( $n = 30$ )	Plumbagin	30.0 $\pm$ 1.5 (5.0)	10.3 $\pm$ 0.7 (6.8)	41.5 $\pm$ 1.8 (4.3)	103

<sup>a</sup> Amounts of the naphthoquinones per gram of dry weight.

<sup>b</sup> Results expressed as a mean  $\pm$  S.D. (CV %).

<sup>c</sup> Internal standard (quinone) was added.

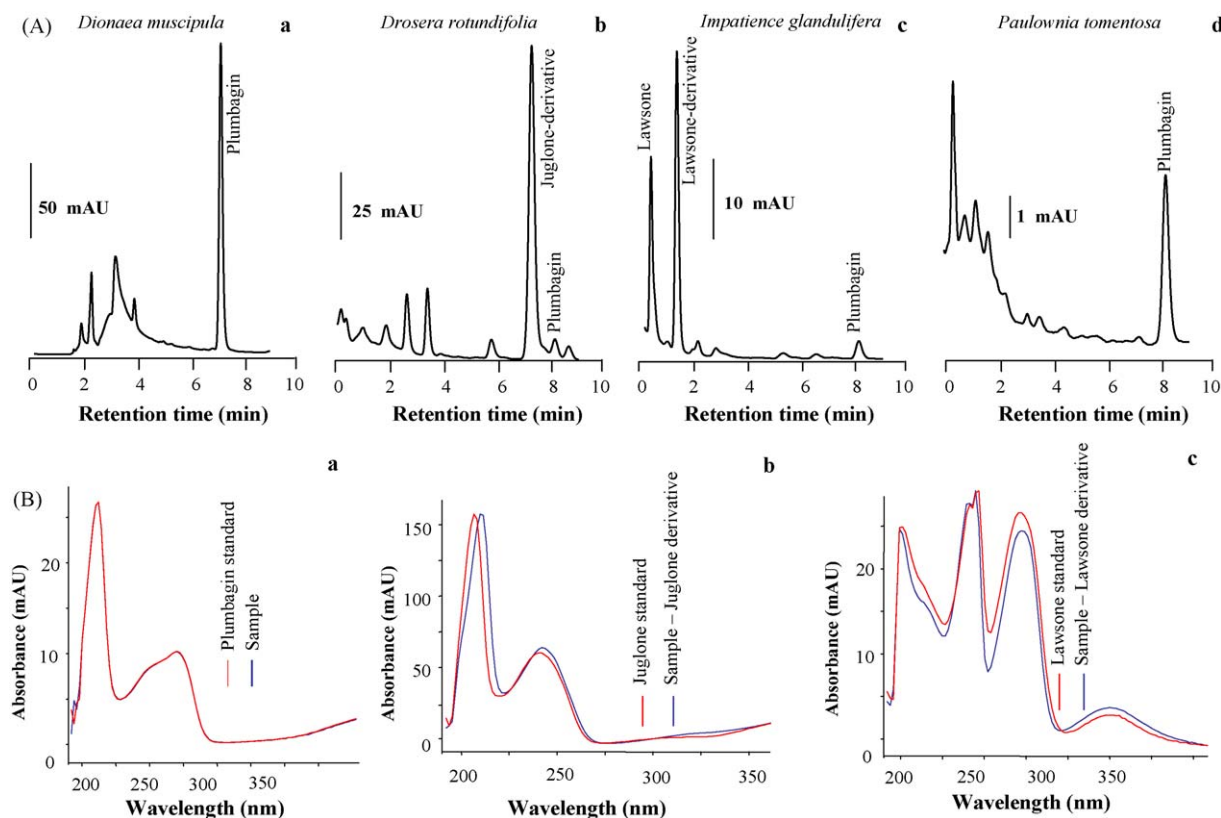


Fig. 4. HPLC-DAD chromatograms of plant samples (A): *Dionaea muscipula* (a), *Drosera rotundifolia* (b), *Impatiens glandulifera* (c) and *Paulownia tomentosa* (d). Diode array spectrums (B) of plumbagin standard and a sample (a), of juglone standard and a sample (b) and of lawsone standard and a sample (c). For other details see Fig. 2 and Section 2.

*tosa* are shown in Fig. 4Aa–d, respectively. The amounts of naphthoquinones detected in plant samples are shown in Table 4. To be specific, in *D. rotundifolia* we detected plumbagin (DAD spectrum is shown in Fig. 4Ba) and a compound, which we called a juglone-derivative, because the DAD spectrum of this compound is very similar to that of juglone (Fig. 4Bb). Except for plumbagin and lawsone, we determined that *I. glandulifera* also contained a compound, whose DAD spectrum is very similar to lawsone (Fig. 4Bc). Therefore, we denoted this compound as a lawsone derivative. A detailed identification of the “derivative” compounds will be published elsewhere. In addition, we have

not evaluated the possibility of interference of isoflavonoids or some other phenolics in our assay [38], because we evaluated selectivity of the analysis by standard additions and by comparing of diode array spectrums obtained with standards.

### 3.3. Changes in content of plumbagin in *D. muscipula* with increasing pH of cultivation medium

A high content of naphthoquinones has been determined in a number of plant species including carnivorous plants [39]. Moreover, the reason, why these plants contain such a high concentration of naphthoquinones is still unclear. This phenomenon is probably correlated with an environment where the plant grows and has to survive [40]. That is why we wanted to study changes in content of naphthoquinones in *D. muscipula* plants according to different pH of cultivation medium. As mentioned above, we determined only plumbagin in *D. muscipula* plants and, therefore, we focused on the content of this naphthoquinone. Primarily we studied the morphological changes of *D. muscipula* plants caused by increasing pH of the cultivation medium. It is evident that the optimal pH of cultivation medium lies between 5 and 8 (Fig. 5A). The lower pH values (2–4) of the cultivation medium caused marked morphological changes in the leaves. In addition, the higher pH values (9–11) caused marked morphological changes in leaves and, moreover, changes in leaf color. Besides that we also determined the dry weight of

Table 4  
Amounts of the naphthoquinones in plant tissue samples ( $n=3$ )

Plant species	Amounts of a naphthoquinone ( $\mu\text{g}$ per gram of dry weight)		
	Lawsone	Juglone	Plumbagin
<i>Juglans regia</i> <sup>a</sup>	–	4980 $\pm$ 160	–
<i>Paulownia tomentosa</i> <sup>a</sup>	–	–	29.1 $\pm$ 0.9
<i>Impatiens glandulifera</i>	539 $\pm$ 14	–	41.2 $\pm$ 1.4
<i>Impatiens parviflora</i>	254 $\pm$ 5	–	45.4 $\pm$ 1.5
<i>Drosera rotundifolia</i>	–	–	22.3 $\pm$ 0.7
<i>Drosera spathulata</i>	–	–	19.6 $\pm$ 0.6
<i>Drosera capensis</i>	–	–	11.1 $\pm$ 0.3
<i>Dionaea muscipula</i>	–	–	8267 $\pm$ 200

<sup>a</sup> Leaves were the analyzed plant tissue.

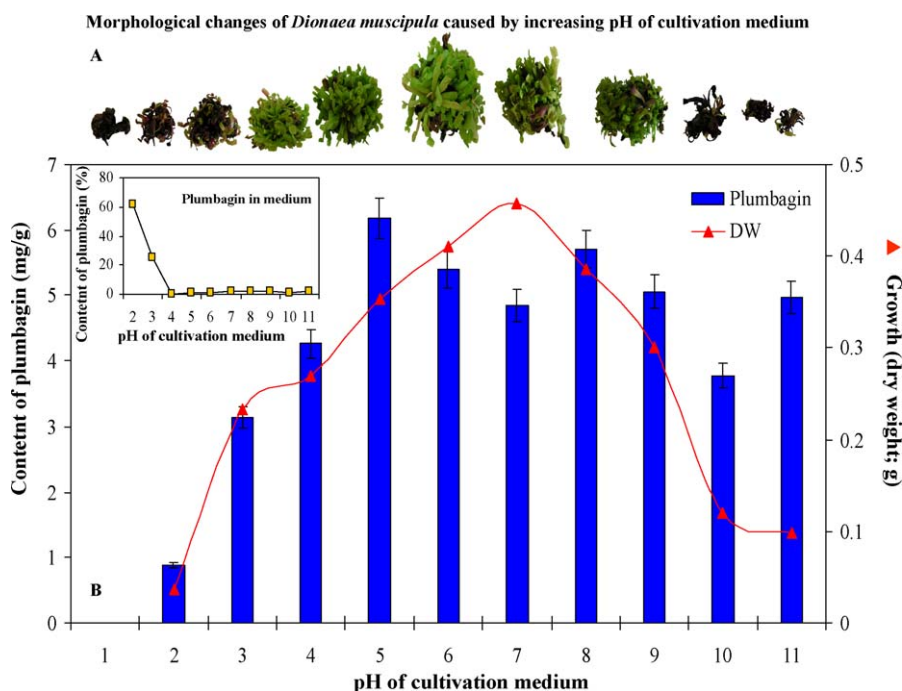


Fig. 5. Morphological changes of *Dionaea muscipula* caused by increasing pH of cultivation medium (A). Dependence of dry weight and plumbagin content in *Dionaea muscipula* plants on different pH of cultivation medium (B). In inset: content of plumbagin in the cultivation medium. For other details see Fig. 2 and Section 2.

the studied plants (Fig. 5B, red line). It clearly follows from the obtained results that the plants cultivated at pH 7 had the highest dry weight and were very well developed without morphological changes (Fig. 5A). After studying the morphological changes of *D. muscipula* plants, we determined the content of plumbagin. The content increased with increasing pH up to 5 and, then, changed gradually (Fig. 5B). The lower content of plumbagin at lower pH values was most interesting to us. Therefore, we determined the content of this naphthoquinone in the cultivation medium, what has not been studied before. We found out that the lower content of plumbagin correlates with the secretion of this naphthoquinone into the cultivation medium (inset in Fig. 5B). The content of plumbagin in the cultivation medium with pH values higher than 3 was negligible.

#### 4. Conclusions

Secondary metabolites level monitoring of naphthoquinones is helpful to obtain quantitative information about the distribution and expression of these compounds in plants during cultivation at different conditions. Here, we optimized and used HPLC-DAD to simultaneously determine of naphthoquinones in different plant species. Plumbagin was determined in almost all of the analyzed samples except *J. regia*, where we quantified milligrams of juglone per gram. Moreover, we studied the changes in the content of plumbagin in *D. muscipula* plants with regard to pH of cultivation medium. We found that the carnivorous plant secretes plumbagin at lower pHs (2–3) of the cultivation medium, which probably relates to their ability to survive in an environment.

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#### References

- [1] A.G. Medentsev, V.K. Akimenko, *Phytochemistry* 47 (1998) 935.
- [2] S.M. Zhong, P.G. Waterman, J.A.D. Jeffreys, *Phytochemistry* 23 (1984) 1067.
- [3] M.B. Zakaria, J.A.D. Jeffreys, P.G. Waterman, S.M. Zhong, *Phytochemistry* 23 (1984) 1481.
- [4] B. Dinda, S.K. Das, A.K. Hajra, *Indian J. Chem. Sect. B* 37 (1998) 672.
- [5] R.G. Binder, B.M.E.R.A. Flath, *Phytochemistry* 28 (1989).
- [6] S.B. Moore, N.J. Hopke, *Chem. Biochem.* 2 (2001) 35.
- [7] J. Bruneton, *Pharmacognosy, Phytochemistry, Medicinal Plants*, England UK, Andover, 1995.
- [8] P. Premakumari, K. Rathinam, G. Santhakumari, *Ind. J. Med. Res.* 65 (1977) 829.
- [9] R.A. Baker, J.H. Tatum, S. Nemeč, *Phytopathology* 71 (1981) 951.
- [10] S. Nemeč, R.A. Baker, J.H. Tatum, *Soil Biol. Chem.* 20 (1988) 493.
- [11] J. Marcinkowska, J.M. Kraft, L.Y. Marquis, *Can. J. Plant Sci.* 62 (1982) 1027.
- [12] R.A. Baker, J.H. Tatum, S. Nemeč, *Mycopathologia* 111 (1990) 9.
- [13] I. Kurobane, N. Zaita, A. Fukuda, *J. Antibiot.* 39 (1986) 205.
- [14] J.H. Tatum, R.A. Baker, R.E. Berry, *Phytochemistry* 26 (1987) 2499.
- [15] J.L. Vennerstrom, J.W. Eaton, *J. Med. Chem.* 31 (1988) 1269.
- [16] P.A.L. Ferraz, F.C. de Abreu, A.V. Pinto, V. Glezer, J. Tonholo, M.O.F. Goulart, *J. Electroanal. Chem.* 507 (2001) 275.
- [17] T.J. Monks, R.P. Hanzlik, G.M. Cohen, D. Ross, D.G. Graham, *Toxicol. Appl. Pharm.* 112 (1992) 2.

- [18] M.M. Gupta, R.K. Verma, G.C. Uniyal, S.P. Jain, *J. Chromatogr.* 637 (1993) 209.
- [19] M. Girzu, D. Fraisse, A.P. Carnat, A. Carnat, J.L. Lamaison, *J. Chromatogr. A* 805 (1998) 315.
- [20] F.C. de Abreu, J. Tonholo, O.L. Bottecchia, C.L. Zani, M.O.F. Goulart, *J. Electroanal. Chem.* 462 (1999) 195.
- [21] M.O.F. Goulart, P. Falkowski, T. Ossowski, A. Liwo, *Bioelectrochemistry* 59 (2003) 85.
- [22] S.R. de Paiva, L.D. Fontoura, M.R. Figueiredo, J.L. Mazzei, M.A.C. Kaplan, *Quimica Nova* 25 (2002) 717.
- [23] B. Klejdus, R. Mikelova, V. Adam, J. Zehnalek, J. Vacek, R. Kizek, V. Kuban, *Anal. Chim. Acta* 517 (2004) 1.
- [24] B. Klejdus, J. Vacek, V. Adam, J. Zehnalek, R. Kizek, L. Trnkova, V. Kuban, *J. Chromatogr. B* 806 (2004) 101.
- [25] R. Causon, *J. Chromatogr. B* 689 (1997) 175.
- [26] R. Bugianesi, M. Serafini, F. Simone, D.Y. Wu, S. Meydani, A. Ferro-Luzzi, E. Azzini, G. Maiani, *Anal. Biochem.* 284 (2000) 296.
- [27] D. Potesil, J. Petrlova, V. Adam, J. Vacek, B. Klejdus, J. Zehnalek, L. Trnkova, L. Havel, R. Kizek, *J. Chromatogr. A* 1084 (2005) 134.
- [28] B. Klejdus, R. Mikelova, J. Petrlova, D. Potesil, V. Adam, M. Stiborova, P. Hodek, J. Vacek, R. Kizek, V. Kuban, *J. Chromatogr. A* 1084 (2005) 71.
- [29] B. Klejdus, R. Mikelova, J. Petrlova, D. Potesil, V. Adam, M. Stiborova, P. Hodek, J. Vacek, R. Kizek, V. Kuban, *J. Agric. Food Chem.* 53 (2005) 5848.
- [30] B. Klejdus, J. Petrlova, D. Potesil, V. Adam, R. Mikelova, J. Vacek, R. Kizek, V. Kuban, *Anal. Chim. Acta* 520 (2004) 57.
- [31] B. Klejdus, J. Zehnalek, V. Adam, J. Petrek, R. Kizek, J. Vacek, L. Trnkova, R. Rozik, L. Havel, V. Kuban, *Anal. Chim. Acta* 520 (2004) 117.
- [32] R. Kizek, M. Masarik, K.J. Kramer, D. Potesil, M. Bailey, J.A. Howard, B. Klejdus, R. Mikelova, V. Adam, L. Trnkova, F. Jelen, *Anal. Bioanal. Chem.* 381 (2005) 1167.
- [33] R. Kizek, L. Trnkova, E. Palecek, *Anal. Chem.* 73 (2001) 4801.
- [34] M. Masarik, R. Kizek, K.J. Kramer, S. Billova, M. Brazdova, J. Vacek, M. Bailey, F. Jelen, J.A. Howard, *Anal. Chem.* 75 (2003) 2663.
- [35] R.K. Gilpin, L.A. Pachla, *Anal. Chem.* 77 (2005) 3755.
- [36] A. Marston, K. Hostettmann, *J. Chromatogr.* 295 (1984) 526.
- [37] B. Hazra, M. Das Sarma, U. Sanyal, *J. Chromatogr. B* 812 (2004) 259.
- [38] L. Marczak, A. Kawiak, E. Lojkowska, M. Stobiecki, *Phytochem. Anal.* 16 (2005) 143.
- [39] U.V. Mallavadhani, G. Sahu, J. Muralidhar, *Pharm. Biol.* 40 (2002) 508.
- [40] L. Adamec, *Biol. Plantarum* 49 (2005) 247.