

Full Paper

Flow Injection Analysis Coupled with Carbon Electrodes as the Tool for Analysis of Naphthoquinones with Respect to Their Content and Functions in Biological Samples

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Abstract: Naphthoquinones are one of the groups of secondary metabolites widespread in nature, where they mostly appear as chromatic pigments. They embody broad-range of biological actions from phytotoxic to fungicidal. 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. The main aim of this work was to suggest a technique suitable to determine lawsone, juglone and/or plumbagin in biological samples and to study of their influence on BY-2 tobacco cells. The BY-2 tobacco cells were cultivated in the presence of the naphthoquinones of interest ($500 \mu\text{g.l}^{-1}$) for 24 h and then the morphological changes were observed. We found out that naphthoquinones triggered the programmed cell death at BY-2 cells, which can be confirmed by the apoptotic bodies in nucleus. After that we suggested and optimized different electrochemical techniques such differential pulse

voltammetry (DPV) coupled with hanging mercury drop (HMDE) and carbon paste electrode, micro flow device coupled with carbon screen printed electrodes and flow injection analysis coupled with Coulochem III detector to determine them. The detection limits of naphthoquinones of interest were expressed as 3S/N and varied from units to hundreds of ng per millilitres according to methods used. Moreover, we utilized DPV coupled with HMDE and micro flow device to determine content of juglone in leaves Persian walnut (*Juglans regia*). We determined that the leaves contained juglone tenths of g per 100 g of fresh weight. The results obtained show the convincing possibilities of using of these methods in analysis of plant secondary metabolites.

Keywords: Naphthoquinones; Plumbagin; Lawsone; Juglone; BY-2 tobacco cells (*Nicotiana tabacum*); Persian walnut (*Juglans regia*); Sensors; Flow analysis; Differential pulse voltammetry; Electrochemical detection; Carbon electrodes.

1. Introduction

Naphthoquinones belong to group of allelopathic metabolites widespread in nature. They have been found in higher plants such as *Plumbaginaceae*, *Juglandaceae*, etc. [1-4], fungi (*Marasmius gramineum* and *Verticillium dahliae*) [5] and microorganisms (*Streptomyces* and *Fusarium*) [6]. Juglone, lawsone and plumbagin are ones of their mostly investigated representatives. Juglone (5-hydroxy-1,4-naphthoquinone) is the characteristic compound of *Juglans* spp., which is reported to occur in fresh walnut leaves [7,8]. Nevertheless, because of polymerization phenomena, juglone is reported to occur in the drug (dry leaves) only in vestigial amounts, which means that the compound is not suitable for use in the quality control of the dry plant [8].

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a naturally occurring yellow pigment derivative from the herb *Plumbago zeylanica* L., a semi-climbing subshrub distributed in thickets or grassland. The whole plant and its root have been used as a folk medicine for the treatment of rheumatic pain, menostasis, carbuncle, and injury due to bumping [9]. Plumbagin has been shown to have anticancer, anti-leishmanial, anti-bacterial and anti-fungal properties [10-12], as well as a contraceptive effect [9]. Moreover plumbagin suppresses NF-kappa B activation and NF-kappa B-regulated gene products through modulation of p65 and I kappa B alpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents [13].

The active dye ingredient, lawsone (2-hydroxy-1,4-naphthoquinone), which constitutes about 1% of the dry weight of the leaves of *Lawsonia alba*, has been implicated in the causation of henna-induced hemolytic anemia because of its structural similarity to other *ortho*-substituted 1,4-naphthoquinones, such as menadione (2-methyl-1,4-naphthoquinone), that are known to induce oxidative injury within red cells [14]. 1,4-Naphthoquinones are thought to induce oxidative damage as a consequence of their ability to undergo redox cycling [15], with the generation of reactive oxygen species.

High performance liquid chromatography with spectrometric detection and electrochemical techniques are the most commonly used method for these purposes [9,16-21]. On the other, electrochemical sensor has not been suggested yet for determination of the lawsone, juglone and/or

plumbagin. The main aim of this work was to suggest a technique suitable to determine lawsone, juglone and/or plumbagin in biological samples. For these purposes, we optimized both stationary and flow electrochemical techniques using carbon electrodes as sensors. Most of all, we aimed on using of carbon screen printed electrodes. Besides that we investigated the influence of the compounds of interest on BY-2 tobacco cells and determined their content in Persian walnut (*Juglans regia*).

2. Materials and methods

2.1. Chemicals

Naphthoquinones (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 ($100 \mu\text{g}\cdot\text{ml}^{-1}$) 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 FIA analysis. The pH value was measured using WTW inoLab Level 3 (MultiLab Pilot; Weilheim, Germany) controlled by a personal computer program (MultiLab Pilot).

2.2. Electrochemical measurements

Differential pulse voltammetry (DPV). The electrochemical analysis of naphthoquinones were performed with AUTOLAB Analyser (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrodes were a hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 and/or carbon paste electrode. The reference electrode was an Ag/AgCl/3M KCl electrode and the auxiliary electrode was a graphite electrode. For smoothing and baseline correction the software GPES 4.4 supplied by EcoChemie was employed. Phosphate buffer (0.2 M) was used as supporting electrolyte. The measurement was carried out in potential range from -0.6 to 0 V with potential step $5 \text{ mV}\cdot\text{s}^{-1}$ and pulse amplitude of 25 mV . The phosphate buffer (0.2 M) was deoxygenated prior the measurement. Prior to analysis of naphthoquinones on carbon paste electrode DPV parameters were used as follows: potential range from 0 to 1.2 V with potential step $5 \text{ mV}\cdot\text{s}^{-1}$ and pulse amplitude 25 mV . All measurements were performed at room temperature.

Preparation of carbon-paste electrode. The carbon paste (about 0.5 g) was made of graphite powder (Aldrich) and mineral oil (Sigma; free of DNase, RNase, and protease). The ratio of the graphite powder and mineral oil was $70/30$ (w/w). This paste was housed in a Teflon body having a 2.5-mm -diameter disk surface. Prior to measurements, the electrode surface was renewed by polishing with a soft filter paper. Then, the surface was ready for measurement of a sample volume of $5 \mu\text{l}$ [22,23].

Micro Flow system. The one channel peristaltic pump PP10 consists of miniature motor with planetary gear and stainless body of peristaltic pump (BVT technologies, Brno). The flow was regulated by PC software (BVT technologies, Brno). The measurement was carried out in flow cells FC2 (BVT Technologies, Brno). The measuring electrodes were home made at Technical University of

Brno, particularly carbon working electrode, silver electrode as auxiliary and Ag/AgCl as reference ones. The flow system was connected with AUTOLAB Analyser (EcoChemie). All measurements were performed at room temperature.

2.3. Flow injection analysis with amperometric detection

An FIA-amperometric system consisted of solvent delivery pump operating in range of 0.001-9.999 ml.min⁻¹ (Model 583 ESA Inc., Chelmsford, MA, USA), a guard cell (Model 5020 ESA, USA), a reaction coil (1 m) and an electrochemical detector. The electrochemical detector (ED) includes one low volume flow-through analytical cells (Model 5040, ESA, USA), which is consisted of glassy carbon working electrode, palladium electrode as reference electrode and auxiliary carbon electrode, and Coulochem III as a control module. The sample (5 µl) was injected manually. The obtained data were treated by CSW 32 software. The experiments were carried out at room temperature. Guard cell potential was 0 V. A glassy carbon electrode was polished mechanically by 0.1 µm of alumina (ESA Inc., USA) and sonicated at the room temperature for 5 min using a Sonorex Digital 10 P Sonicator (Bandelin, Berlin, Germany) at 40 W.

2.4. Plant materials

BY-2 tobacco cell suspension. A suspension culture of *Nicotiana tabacum* BY-2 line was grown in liquid Murashige and Skoog medium supplemented with sucrose (30 g.l⁻¹), KH₂PO₄ (0.2 g.l⁻¹), thiamine (1 mg. l⁻¹) and 2,4-dichlorophenoxyacetic acid (0.2 mg.l⁻¹), according to Nagata [24]. The suspension cultures (20 ml) were grown in 50 ml Erlenmeyer flasks at 27°C with shaking at 135 rpm (Kühner Shaker, type: LT-W, Adolf Kühner AG, Switzerland). Subcultivation was performed after 3 or 4 days by transferring 2 or 1 ml, respectively, of suspension culture into a fresh medium (total volume 20 ml).

Juglans regia. The leaves of Persian walnut (*Juglans regia*) were harvested in Botanic garden of Mendel University of Agriculture and Forestry in Brno, October 2005 and July 2006. The harvested leaves (0.10 g ± 5 mg), 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 FIA system and 100 times diluted with ACS water.

2.5. Cytological analysis

The sample of tobacco cell suspension was mixed with PEM buffer (100 mM PIPES, 10 mM EGTA, 10 mM MgCl₂, pH 6.9) containing 4 % of formaldehyde in ratio 1:1. After the incubation (30 min., 25°C) the fixed cells were three times washed with PEM buffer and resuspended in PEM buffer with 0.1 % Triton X100 and 1 µg.ml⁻¹ Hoechst 22385. The nucleus morphology was observed by the fluorescent microscope (Olympus AX 70) using the broad band UV excitation (cube U-MWU).

2.6. Statistical analysis

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

3. Results and discussion

Although naphthoquinones have been intensively studying for more than forty years [25], their biochemical mechanism of action is still unclear. It is a common knowledge that they can enter and influence a cell cycle [10,26-32]. Due to this their effects on tumour cell lines like such as P 338, BG-1 and HL-60 have been investigated [33]. Here we aimed on suggesting of a sensor for determination of certain naphthoquinones (lawsone, plumbagin and juglone, see in Fig. 1A) and on investigating of their effects on BY-2 tobacco cells. The effects of these substances on whole plants have been described but their influence on plant cell suspension is not clear yet, whereas BY-2 tobacco cells are very suitable for these purposes.

3.1. Morphological changes of BY-2 tobacco cells caused by naphthoquinones

The BY-2 tobacco cells were cultivated in the presence of the naphthoquinones of interest ($500 \mu\text{g}\cdot\text{l}^{-1}$) for 24 h and then the morphological changes were observed. The cell nuclei of non-treated BY-2 tobacco cells were well observable and their cytoplasm fluoresced weakly greenly (Fig. 1B). On the other hand the treated BY-2 cells embodied a number of morphological changes. The cell cytoplasm fluoresced intensively in comparison with non-treated ones (Fig. 1C-G), which is probably associated to the increase of activity of intracellular esterases [34-36]. The marked changes of chromatin of treated BY-2 cells were observed. As we observed in the microphotography, the nucleus pre-apoptotic changes, particularly, the strong initiation condensation of chromatin on nuclear periphery was observed (Fig. 1D, G). In addition, we found out that naphthoquinones triggered the programmed cell death at BY-2 cells, which can be confirmed by the apoptotic bodies in nucleus (Fig. 1E,F) [37,38]. This effect could be explained by the ability of naphthoquinones to generate the reactive oxygen species, the potential depletion on mitochondrial membranes and the inhibition of topoisomerases I and II [5,10,39]. Moreover, plumbagin is a potent inhibitor of the NF-kappa B activation pathway that leads to suppression of NF-kappa B-regulated gene products [13]. Thanks to properties of the naphthoquinones, the interest suggesting and optimizing of easy-to-use and sensitive determination of them increases.

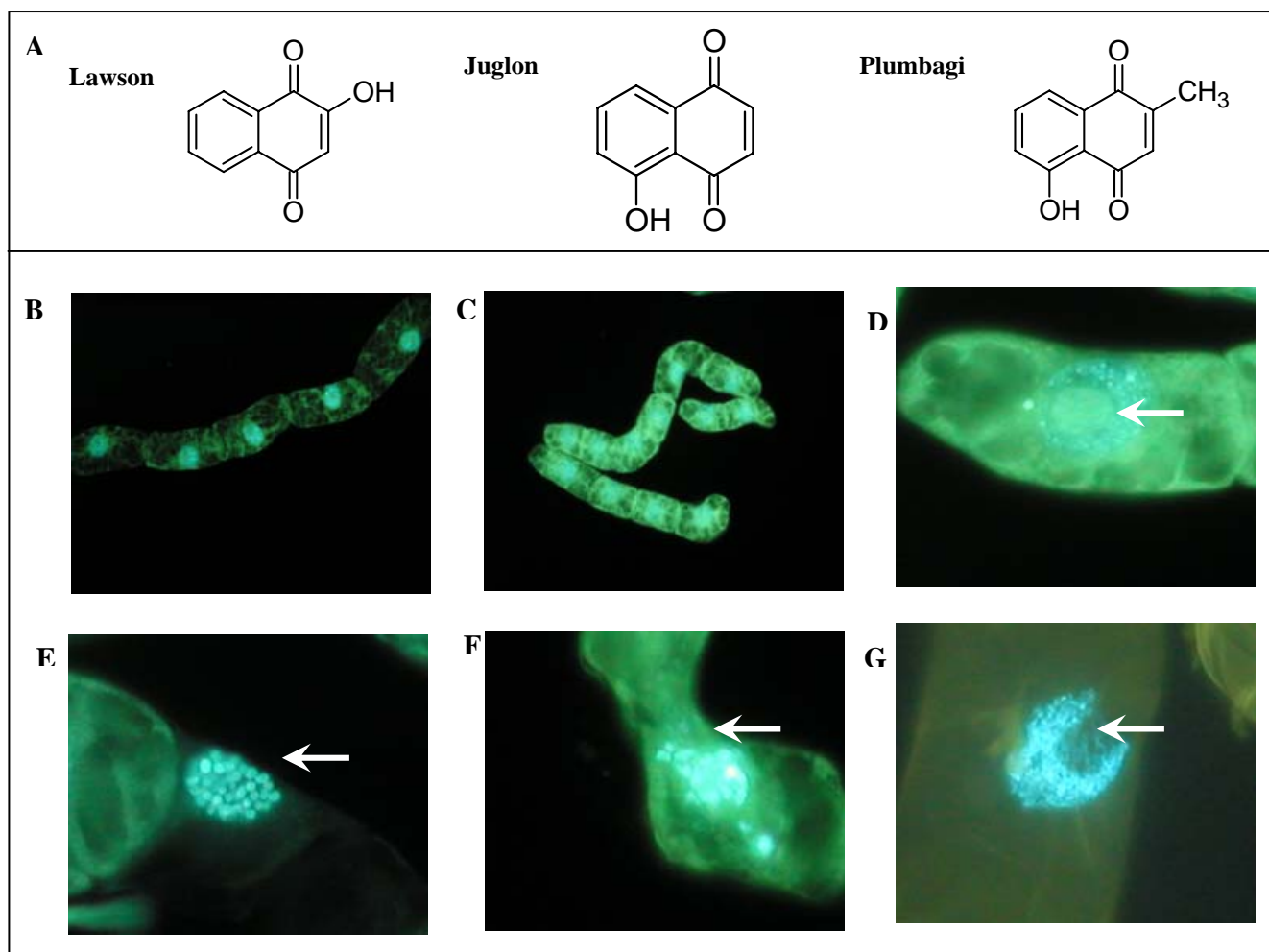


Figure 1. (A) Chemical structures of lawsone, juglone and plumbagin. The morphological changes of BY-2 cells induced by the naphthoquinones juglone and plumbagin. The tobacco cells were fixed and dyed with fluorescent dye Hoechst 33258 and observed by epifluorescent microscopy. (B) Control, non-treated BY-2 cells. (C-G) The tobacco cells treated by plumbagin and juglone ($500 \mu\text{g}\cdot\text{l}^{-1}$) for 24 hours; (C) overall insight; (D) beginning chromatin condensation on the nuclear periphery – pre-apoptotic nucleus; (E) apoptotic nucleus – plumbagin; (F) apoptotic nucleus – juglone; (G) – nucleus with strong chromatin condensation. The arrows show on described phenomenon. Magnification of the figures: (B-C) $\times 400$ and (D-F) $\times 1000$.

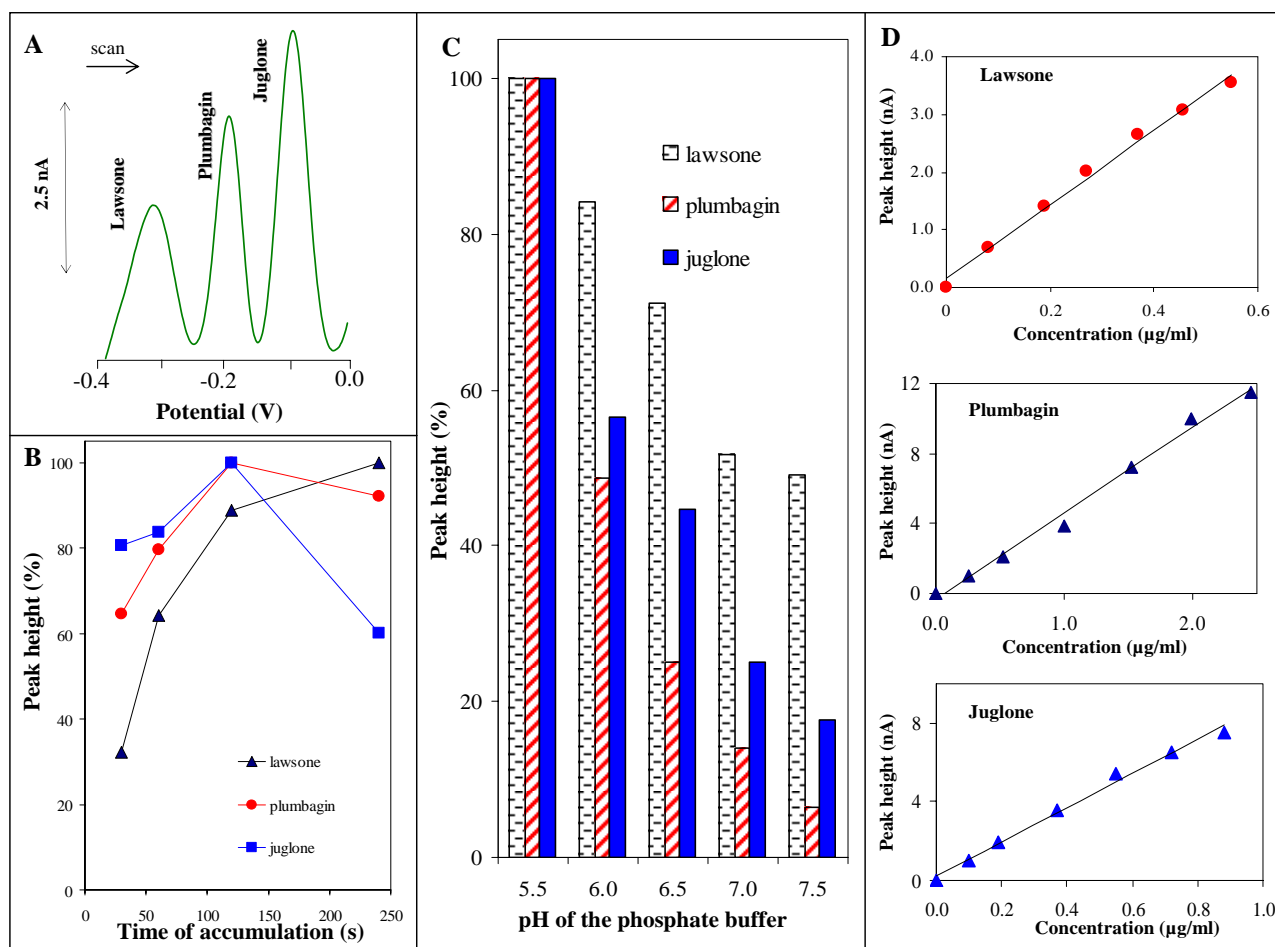


Figure 2. Naphthoquinone analysis by stationary electrochemical method. (A) The DP voltammogram of lawsone, juglone and plumbagin ($1 \mu\text{g}\cdot\text{ml}^{-1}$) simultaneously determined on the surface of HMDE. The change of signal height of the naphthoquinones with increasing (B) time of accumulation and (C) pH of phosphate buffer – supporting electrolyte. Signal heights of 7 nA for lawsone, of 4 nA for plumbagin and of 8 nA for juglone correspond to the 100%. (D) The dependence of naphthoquinone peak height on its concentration. The naphthoquinones were measured in the presence phosphate buffer (0.2 M) in potential range from -0.6 to 0 V with potential step $5 \text{ mV}\cdot\text{s}^{-1}$ and pulse amplitude 25 mV. For other details see “Materials and Methods” section.

3.2. Electrochemical determination of naphthoquinones

The higher attention was given to chromatographic methods of naphthoquinones determination [18,40-45]. The electrochemical techniques have been utilizing mainly to study of naphthoquinones chemical interaction [46-48], but their utilizing to naphthoquinone determination in biological samples has not been published yet. Here, we focused primarily to study of lawsone, plumbagin and juglone behaviour on mercury drop electrode measured in the presence of phosphate buffer (pH 6.0). We obtained the characteristic voltammograms with electrochemical signals corresponds to electrochemical changing of compounds of interest. Due to electroanalytical purposes, the characteristic signals at potential of -0.37 V for lawsone, of -0.23 V for plumbagin and of -0.04 V for juglone were chosen. Moreover, we were able to determine the naphthoquinones of interest

simultaneously (Fig. 2A). During simultaneous determination the slight potential shift of single naphthoquinones signals was observed ($\pm 5\%$).

It clearly follows from the results obtained that the naphthoquinones can interact strongly with the surface of HMDE. Particularly, we observed increase in the height of juglone and plumbagin signal with increasing time of accumulation up to t_A of 120 s and then the signal decreased. As for lawsone, its signal increased more than 60 % at t_A of 300 s in comparison with the signal measured at t_A of 0 s and then the signal increased slowly (Fig. 2B). It clearly follows from the results that the time of accumulation of 120 s was the most suitable for simultaneous determination of the naphthoquinones (Fig. 2B). It is a common knowledge that the pH of the supporting electrolyte influences markedly the height of a signal. It clearly follows from the results obtained that the highest signals of the naphthoquinones of interest were observed at pH 5.5. The electrochemical signals decreased at higher pH values, mostly at the plumbagin and at the least at lawsone (Fig. 2C). After the optimizing step, measuring the dependence of the naphthoquinone electrochemical signal on its concentration followed (Fig. 2D). The calibration curves obtained were linear within the tested concentration range from 0.1 to 5 $\mu\text{g}\cdot\text{ml}^{-1}$. The detection limits were expressed as 3S/N and varied from 5 to 100 $\text{ng}\cdot\text{ml}^{-1}$ with relative standard deviations about 3% (Table 1).

Consequently, the suggested method was utilized to study the naphthoquinones content directly in plant material. The leaves of Persian walnut (*Juglans regia*) was chosen as plant material analysed [16]. This tree was chosen not only because it's multilateral usage, but also for the high naphthoquinones level, especially juglone and plumbagin. We determined that the leaves contained plumbagin at 320 $\mu\text{g}\cdot\text{ml}^{-1}$ and juglone at 290 $\mu\text{g}\cdot\text{ml}^{-1}$, which corresponds to the naphthoquinone content of 0.3 and 0.2 g per 100 g of fresh weight.

Even despite the number of interfering compounds like carboxylic acids (linolic, linolenic and caffeic acid), terpens (germakren D), vitamins (provitamin A, some vitamins B, vitamin C and E) and not least tannins and their derivatives (galic and egalic acid) the naphthoquinones were easily detectable by the suggested technique in the leaves of Persian walnut.

Table 1. Differential pulse voltammetric characteristics of the naphthoquinones of interest ($n = 5$).

Naphthoquinone	Equation ^a	R ²	LOD (nM) ^c	LOQ (nM) ^d	R.S.D. (%) ^e
Lawsone	$y = 6.4353x + 0.1462^b$	0.9921 ^b	5	16	3.5
Plumbagin	$y = 4.8977x - 0.3042^b$	0.9922 ^b	100	333	2.8
Juglone	$y = 8.7097x + 0.2237^b$	0.9921 ^b	18	60	2.9

^a ... The concentration range was from 0.1 to 5 μM of the compound of interest.

^b ... The equation was derived from the dependence of the peak height on the naphthoquinone concentration.

^c ... Limit of detection is expressed as 3 S/N.

^d ... Limit of quantification is expressed as 10 S/N.

^e ... Relative standard deviation.

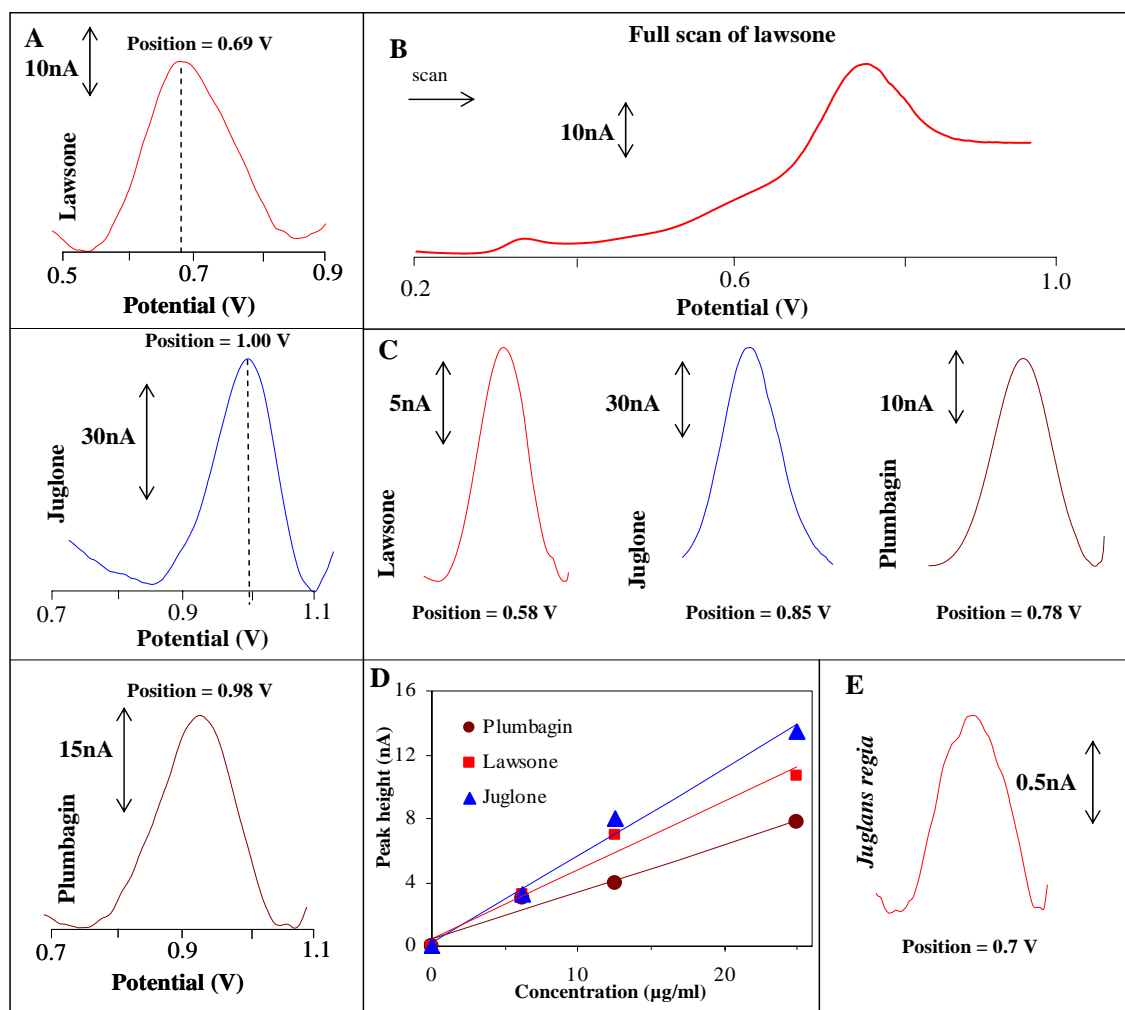


Figure 3. Carbon paste electrode. (A) Baseline corrected DP voltammograms of lawsone, juglone and plumbagin ($1 \mu\text{g}\cdot\text{ml}^{-1}$) measured on the surface of carbon paste electrode. DPV parameters were used as follows: potential range from 0 to 1.2 V with potential step $5 \text{ mV}\cdot\text{s}^{-1}$ and pulse amplitude 25 mV. Carbon screen printed electrode. (B) Full scan of Lawsone measured on carbon screen printed electrode using micro flow device. (C) Baseline corrected voltammograms of lawsone, juglone and plumbagin ($1 \mu\text{g}\cdot\text{ml}^{-1}$) on measured on the surface of carbon screen printed electrodes. (D) Dependence of signal height on the concentration of single naphthoquinone. (E) Voltammogram of the real sample of walnut leave 100 times diluted. Pumping of the supporting electrolyte (0.2 M phosphate buffer, pH 5.5) by using the micro pump (140 s, 5 000 rpm), the samples were injected each 30 s from stock tube (5 000 rpm). For other details see “Materials and Methods” section.

3.3. Electrochemical analysis of naphthoquinones using a carbon paste electrode

The mercury electrode can not be utilized for the analysis in flow arrangement, thus we used the solid electrodes (carbon, gold, etc.) for these purposes. The oxidation signals of naphthoquinones of interest can be observed on voltammograms obtained. These signals after baseline correcting are shown in Fig. 3A [49,50]. The signals of lawsone were observed at the potential of $0.68 \pm 0.03 \text{ V}$ ($n = 5$), of juglone at $1.00 \pm 0.02 \text{ V}$ ($n = 5$) and of plumbagin at $0.98 \pm 0.02 \text{ V}$ ($n = 5$). The peak height of

single naphthoquinone was proportional to its concentration within the range from 1 to 50 $\mu\text{g}\cdot\text{ml}^{-1}$ with the detection limit below 1 $\mu\text{g}\cdot\text{ml}^{-1}$. The details will be published elsewhere.

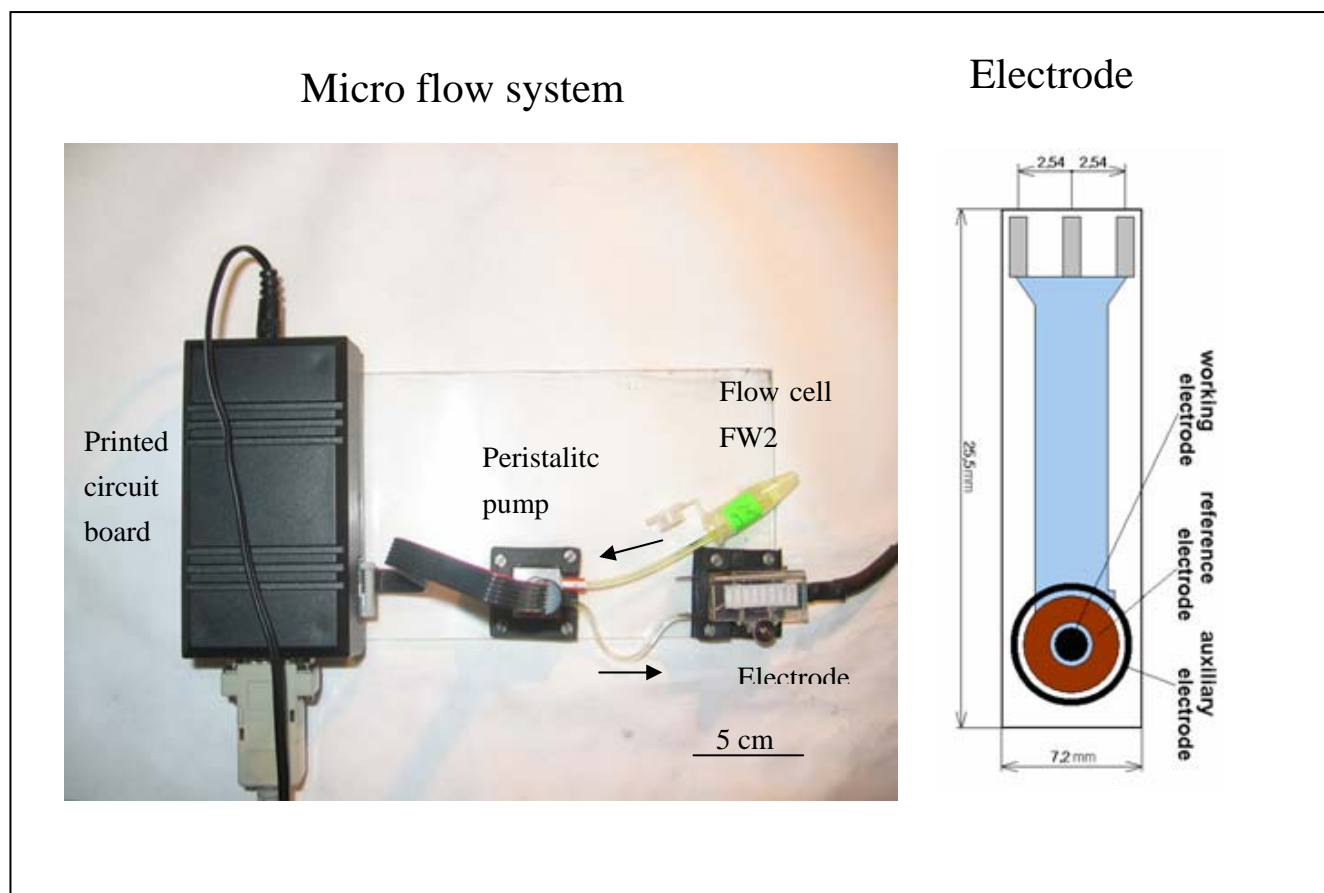


Figure 4. The photography of the micro flow system built from the micro pump, flow cell and printed circuit board. The scheme of the designed printed electrodes (working carbon, auxiliary carbon and referent Ag/AgCl electrode).

3.4. The electrochemical analysis of naphthoquinones using the micro flow system

Due to needs of routine analysis, it is very useful to connect sensitive detection technique with flow instrument [51-54]. For these purposes, the instrument built up from miniaturized peristaltic pump, flow cell and control printed circuit board was used (Fig. 4). The printed electrodes, whose construction is shown in Fig. 4, are placed in the flow cell. Particularly, the working electrode is placed in the middle, around the working electrode is placed the referent (Ag/AgCl) and auxiliary ones. The electrodes is inserted into the slit of cell and tightened by closing of the door. The cell assures the wall-jet flow around the working electrode. The cell contains also the contact and output cable. The micro pump was controlled by software REV (BVT technologies, Brno) and the electrochemical responses were recorded by potentiostat/galvanostat AUTOLAB using the GPES 4.9 software (Eco Chemie).

It is a common knowledge that the flow rate influences the electrochemical response markedly, especially in voltammetric, but also in amperometric analysis [55-59]. Due to this the experimental method of naphthoquinones analysis in flow system was designed. Primarily the supporting electrolyte (phosphate buffer pH 5.5) was pumped into the miniaturized electrochemical system by the micro

pump (140 s, 5 000 rpm), which leads to filling of the electrochemical cell in very small volume of 25 μl . Then, the sample was pumping for 30 s (5 000 rpm). After that the voltammetric analysis followed. Moreover, the electrode can be reused for other analysis after the soft cleaning of its surface with ACS water. In this experimental design, it was possible to run five analyses with the 5-10% decrease of the signal of compound of interest, but the reproducibility of the measurement was still very good. During the analysis very well developed and characteristic oxidative signals of single naphthoquinones were obtained and are shown in Fig 3B. After the application of the baseline correction we were able to distinguish the signals of lawsone at the potential of 0.58 ± 0.02 V ($n = 5$), juglone at 0.85 ± 0.0 V ($n = 5$) and plumbagin at 0.78 ± 0.03 V ($n = 5$) see in Fig. 3C. The shifting of the signals into the positive potential of about 100 mV was observed in comparison with the measurement on carbon paste electrode. These shifts could be associated with the designed electrode system and, above all, with different construction of the referent electrode. In addition, we studied the dependence of the electrochemical signal observed on the concentration of naphthoquinone. The obtained calibration curves were linear within concentration range from 1 to 25 $\mu\text{g}\cdot\text{ml}^{-1}$ (Fig. 3D). The detection limits were expressed as 3S/N and were from 0.15 to 0.35 $\mu\text{g}\cdot\text{ml}^{-1}$ with relative standard deviation of the determination about 4.5 % (Table 2).

Table 2. Analysis of the naphthoquinones by micro flow analysis ($n = 5$).

Naphthoquinone	Equation ^a	R ²	LOD ($\mu\text{g}\cdot\text{ml}^{-1}$) ^b	LOQ ($\mu\text{g}\cdot\text{ml}^{-1}$) ^b	R.S.D. (%) ^b
Lawsone	$y = 0.597x - 0.600^b$	0.9999 ^b	0.28	0.93	4.5
Plumbagin	$y = 0.262x + 0.567^b$	0.9952 ^b	0.35	1.17	4.8
Juglone	$y = 0.567x + 0.038^b$	0.9972 ^b	0.15	0.50	5.1

^a ... The concentration range was from 1 to 25 $\mu\text{g}\cdot\text{ml}^{-1}$.

^b ... The equation was derived from the dependence of the peak height on the naphthoquinone concentration.

^c ... Limit of detection is expressed as 3 S/N.

^d ... Limit of quantification is expressed as 10 S/N.

^e ... Relative standard deviation.

3.5. Determination of juglone in leaves of Persian walnut

Similarly to the naphthoquinones determination in real sample by using the classical electrochemical methods, likewise we analyzed the extract from Persian walnut (*Juglans regia*) by the abovementioned technique (Fig. 3E). Walnut (*Juglans regia* L.) leaf has been widely used in folk medicine for treatment of venous insufficiency and haemorrhoidal symptomatology, and for its antidiarrheic, antihelmintic, depurative and astringent properties [8]. Keratolytic, antifungal, hypoglycaemic, hypotensive, anti-scrofulous and sedative activities have also been described [7].

As for analysis of the leaves, we determined the concentration of plumbagin and juglone by flow analysis on carbon printed electrode (Fig. 4) and found out that the results were 502 $\text{ng}\cdot\text{ml}^{-1}$ and 458 $\text{ng}\cdot\text{ml}^{-1}$, respectively. This corresponds to plumbagin and juglone concentration about 0.5 and 0.4 g per 100 g of fresh weight, respectively. The naphthoquinones content determined in leaves of Persian

walnut by flow electrochemical techniques slightly differs from stationary ones. The observed differences could be associated with plant phenophasis (time of collecting of single samples; October 2005 for DPV HMDE analysis and July 2006 for micro flow device).

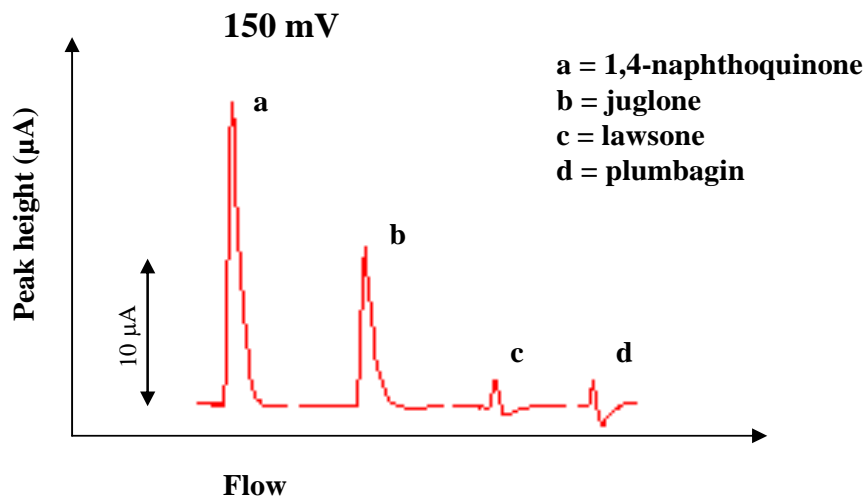


Figure 5. A FIA-amperometric record of the naphthoquinones ($1 \mu\text{g}\cdot\text{ml}^{-1}$) at the potential of 150 mV. The flow rate of the electrolyte (0.2 M phosphate buffer, pH 5.5) was $0.5 \text{ ml}\cdot\text{min}^{-1}$. The sample ($5 \mu\text{l}$) was injected manually. The working electrode was made from glassy carbon, auxiliary carbon electrode and referent hydrogen palladium electrode. For other details see “Materials and Methods” section.

At the end of our work, we attempted to utilize the results obtained from stationary and micro flow analysis of naphthoquinones for suggesting their detection by robust flow injection analysis coupled with Coulochem III electrochemical detector. The FIA-ED record of analysis of juglone, lawsone and plumbagin standards is shown in Fig. 5. It clearly follows from the results obtained that we can successfully utilize the results obtained from micro flow device during analysis by more robust technique.

Conclusion

The electrochemical methods are suitable for simultaneous determination of the compounds of interest not only in a biological sample without separation step but also directly in the biological sample [60-62]. Here, certain naphthoquinones (lawsone, plumbagin and juglone) were sensitively analyzed by using the differential pulse voltammetry coupled with hanging mercury drop and carbon paste electrodes and by injection analysis coupled with carbon printed electrode. The results obtained show the convincing possibilities of using of these methods in analysis of plant secondary metabolites. Thus, the electrochemical methods are convenient for the construction and design of the miniaturized sensors and biosensors [63-67].

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