Int. J. Electrochem. Sci., 7 **(2012)** 908 - 927

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Determination of Eight Polycyclic Aromatic Hydrocarbons and in Pea Plants (*Pisum sativum* L.) Extracts by High Performance Liquid Chromatography with Electrochemical Detection

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Received: 2 July 2011 / *Accepted:* 8 November 2011 / *Published:* 1 February 2012

In the field of plant physiology, knowledge about polycyclic aromatic hydrocarbons (PAHs), mechanisms of actions are still missing and are limited despite the fact that induction of oxidative stress by several PAHs has been demonstrated. Primarily, high performance liquid chromatography coupled with UV detector and high performance liquid chromatography coupled with electrochemical detector were optimized for detection and quantification of the following PAHs as fluoranthene, pyrene, benzo[*a*]pyrene, naphthalene, benzo[*a*]antracene, methylanthracene, triphenylene and coronene. The optimized conditions were as follows: mobile phase - A: acetic acid (50 mM) and B: methanol (100 %). Compounds were eluted by linearly increasing gradient: 0 → 6 min (70 % B), 6 → 10 min (100 % B), 10 → 13 min (100 % B), 13 → 14 min (70 % B), 14 → 20 min (70 % B). Detection was performed at 275 nm (UV detector) and at 900 mV (electrochemical detector). One analysis lasted less than 20 minutes. Detection limits were estimated as 3 S/N down to subunits of μM for HPLC-ED and down to units of μM for HPLC-UV. It clearly follows from the results obtained that HPLC-ED has for more than one order of magnitude lower detection limits compared to HPLC-UV. It can be assumed that this difference would be higher, i.e. electrochemical detector could be more sensitive, if mobile phase with lower content of organic solvents would be used. Based on the above mentioned results, HPLC-ED was used in the following experiments. To test the applicability of our method, pea plants (*Pisum sativum* L. cv. Zazrak) were treated with fluoranthene (FLT) in the concentrations 0, 0.1, 1.0 and 5.0 mg/l and their tissues analysed to determine applied PAHs. Optimised HPLC method

enabled determination of fluoranthene transport into different plant organs - roots, stems and leaves. Hydroponically cultivated pea plants also proved as suitable model for monitoring of effect of fluoranthene on uptake and transport of physiologically crucial elements as calcium, copper, iron and zinc. Significant differences between control and treated plants were determined, however, role of these elements in responses to fluoranthene treatment should be further investigated.

Keywords: Polycyclic Aromatic Hydrocarbons; High Performance Liquid Chromatography with UV and Electrochemical Detection; Fluoranthene; Pea Plants; *Pisum sativum*

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) represent group of highly lipophilic compounds containing aromatic rings. PAHs as pure chemicals are white or yellowish crystalline compounds. These compounds belong to group of the most dangerous pollutants occurring in environment due to anthropogenic activity. Their origin is connected with combustion of fossil fuels and organic matter, mining and ore processing as the group of the most important sources of PAHs [1-8]. Petrochemical industry and manufacturing of paints and lacquers represent the second most important source of PAHs [1,9,10]. Natural sources, such as forest fires and volcanic eruptions contribute also to PAHs releasing into the environment, but the previous mentioned sources represent real threat [11,12]. PAHs are worldwide distributed in air, soils, waters, but also in river and ocean sediments and sludge [13-17]. Due to their abundance, it is not surprising that they are able to entry food chain in every level [18]. PAHs are highly toxic for plants [19] as well as for animals [20,21] in the form of both acute and chronic intoxication depending on time of exposure and concentration of PAHs. Intoxication of animals is connected with hormone imbalance, hepatotoxicity, anaemia and changes in blood count [22]. Smoking represents one of the most important ways of PAHs exposition for mankind [23]. PAHs, especially benzo[*a*]pyrene, are also connected with carcinogenesis and genotoxical effects, which are often associated with formation of electrophilic intermediates and their subsequent binding into nucleophilic parts of DNA under formation of DNA-adducts [24,25]. These adducts can stop cell cycle or alter gene transcription. Due to PAHs lipophilicity, they are deposited in lipophilic organelles, respectively in cells membrane structures both animal, and plant cells. Transport of PAHs within both animal and plant body was demonstrated [26,27]. Negative effects of PAHs including fluoranthene on plants were shown in numerous experimental papers [19,28,29]. They include affecting of processes of photosynthesis and respiration. Oguntimehin et al. demonstrates negative effect of fluoranthene on biomass production [30].

Due to determination of distribution and evaluation of impact of PAHs on the environment, robust analytical methods are needed. An extraction of PAHs from different biological matrixes belongs to the one of the most important and complex steps in PAHs analysis. Soxhlet-based extraction, pressurized liquid extraction, and supercritical fluid extraction together with microwave-assisted extraction, ionic liquid based dispersive liquid-liquid and dispersive solid phase extractions are the most commonly used techniques [31-34]. It has been proved that choice of extraction technique could significantly affect subsequent measurement of a real sample [35]. As detection techniques, high

performance liquid chromatography (HPLC) with UV and/or fluorimetric detection is mostly used [36-39]. In addition to HPLC, gas chromatography coupled with mass spectrometry, various electrochemical biosensors, adsorptive stripping voltammetric determination or electrochromatography with mass detection are also used for PAHs analysis [40-47].

Despite the fact that HPLC methods are widely used for PAHs detection and quantification, there are still possibilities for suggesting, developing and optimizing other analytical techniques and methods (see scheme of HPLC-UV and/or HPLC-ED, Fig. 1A). Rapid, accurate and low-cost analytical methods are developed mainly due to the contamination of the environment by PAHs and necessity of rapid analysis of biological samples. This study was aimed at optimization of HPLC coupled with UV and/or electrochemical detector (Fig. 1A) for simultaneous detection of eight PAHs - fluoranthene, pyrene, benzo[*a*]pyrene, naphthalene, benzo[*a*]anthracene, methylanthracene, triphenylene and coronene (Fig. 1B). These compounds belong to a group of organic pollutants with assumable accumulation in agriculturally important crops with subsequent entry to the food chain [48]. Their effect on numerous physiological, metabolic and biochemical pathways including uptake and transport of essential metal ions as calcium, iron, copper and zinc may be expected. Therefore, we tested the applicability of the method on the analysis of tissues of pea plants (*Pisum sativum* L.) treated with fluoranthene as one of the compound, on which the method was optimized.

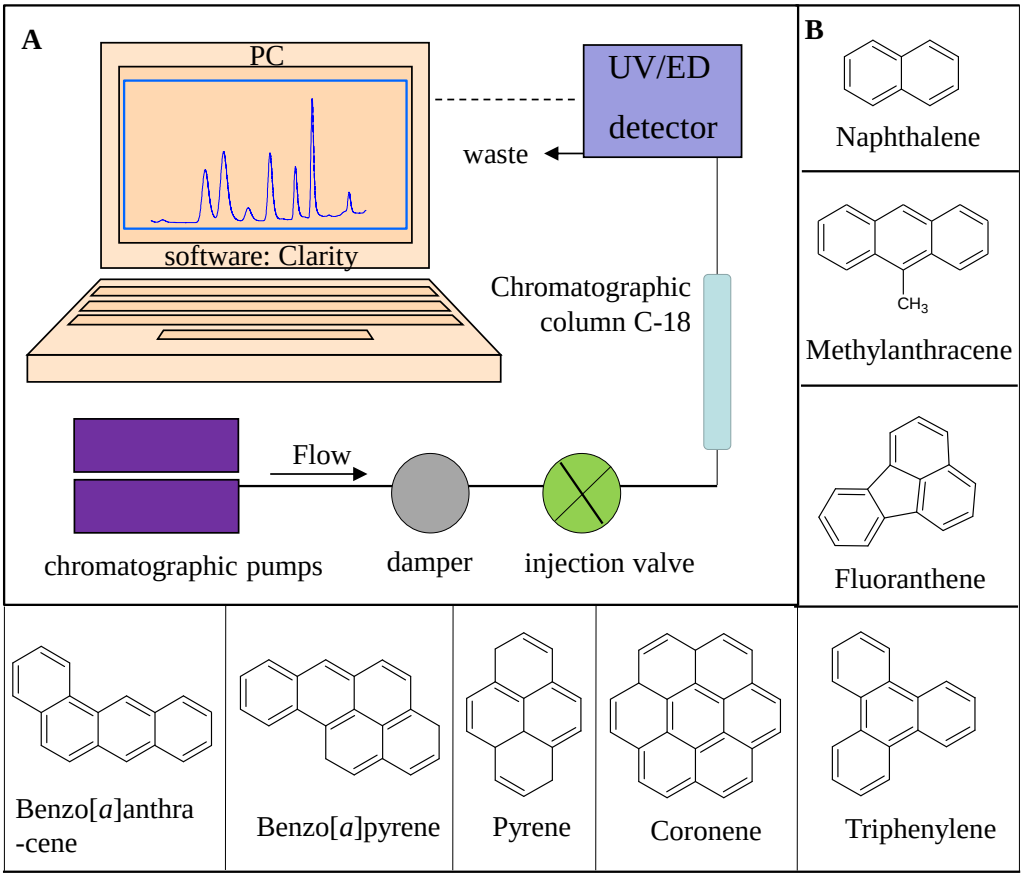

Figure 1. (**A**) Scheme of HPLC-UV instrument. (**B**) Structures of studied substances of fluoranthene, pyrene, benzo[*a*]pyrene, naphthalene, benzo[*a*]anthracene, methylanthracene, triphenylene and coronene.

2. EXPERIMENTAL PART

2.1 Chemicals

Standards of polycyclic aromatic hydrocarbons were purchased from Sigma Aldrich (Sigma Aldrich, USA). The stock standard solutions of PAHs (1 mM) were prepared by dissolution in dimethyl sulphoxide – DMSO (Sigma Aldrich, USA) and were stored in the dark at –20 °C in a freezer. All other chemicals were of ACS purity and were purchased from Sigma Aldrich.

2.2 Cultivation of pea plants

For the experiments, pea plants (*Pisum sativum* L. cv. Zázrak) with the similar stage of the ontological development, morphological characteristics and weight were used. Seeds of pea were germinated and grown in vermiculite in cultivation box under strictly defined conditions: temperature 23±2 °C (light) / 18 °C±1 °C (dark), photoperiod 16/8 (6 – 22 h), light intensity 140 $\mu E/m^{-2}s^{-1}$ and relative humidity 80 %. Five day-old plants were transferred into hydroponic vessels into Hoagland nutrition medium (Sigma Aldrich, USA) adjusted to pH = 6.5 and supplemented by polycyclic aromatic hydrocarbon fluoranthene (FLT, purity 98 %, Sigma-Aldrich, USA) in concentrations 0.1, 1.0 and 5.0 $mg.l^{-1}$. The first group of control plants was cultivated in cultivation medium without FLT supplementation. The second group of control plants was represented by pea plants treated with the highest dose of dimethyl sulphoxide as non-water solvent. Cultivation medium was replaced each five days. Cultivation conditions were as follows: temperature 23±2 °C, photoperiod 16/8 (6 – 22 h), light intensity 140 $\mu E \cdot m^{-2}s^{-1}$. Plants were cultivated for 18 days. In the end of the experiment, plants were harvested, carefully rinsed with distilled water, divided into individual organs and prepared prior to measurements.

2.3 Automated spectrometric measurements

Spectrometric measurements were performed on an automated chemical analyser BS-200 (Mindray, China). Reagents and samples were placed in cooled sample holder tempered to 4 °C and automatically pipetted directly into plastic cuvettes. Mixtures of samples and reagents were incubated at 37 °C. After reagents or sample addition, mixture was consequently stirred by automatic stirrer. Sample contamination was prevented by the washing steps. The washing steps were done with distilled water (18 MΩ) in the midst of the pipetting. Apparatus was operated by a software BS-200 (Mindray, China).

2.3.1 Protein determination – Biuret method

Biuret solution was prepared from 15 mM potassium sodium tartrate (100 mM NaI, 15 mM KI and 5 mM $CuSO_4$). For the determination of the total protein content, albumin (1 $mg.ml^{-1}$ in phosphate buffer, 20 mM, pH 7) was used as a standard. The measurement was carried out as follows: 180 µl of

the Biuret solution was mixed with 45 µl of a sample or standard solution of albumin. A mixture was stirred and incubated for 10 min. at 37°C. Then, an absorbance was measured at 546 nm. All measurements were carried out in triplicates.

2.3.2 Determination of elements

For the determination of selected elements (calcium, iron, copper and zinc), volume of 200 µl of reagent (Groner, Germany) was pipetted into plastic cuvettes. Immediately, sample (20 µl) was added and mixture was stirred by the automatic stirrer. Absorbance was measured continuously for 10 min. at the following wavelengths as λ = 570 nm (copper), λ = 570 nm (zinc), λ = 570 nm (iron) and λ = 605 nm (calcium). To calculate content of the element, both absorbance of reagent and absorbance of reagent with sample after 10 min. incubation were used. Final concentrations of elements were recalculated using calibration curves for individual elements. Their contents were determined in both plants and cultivation media samples.

2.4 Extraction of fluoranthene from plant material for HPLC analysis

Plant sample (0.5 g) was homogenized with mixture of benzene and methanol (3:1, *v*/*v*, 2 ml, both Penta, Czech Republic). After homogenization in grinding mortar, mixture of solvents was evaporated. Immediately after evaporation, 1 ml of mixture of solvents was added and quantitatively transferred into a micro test-tube (volume 1.5 ml Eppendorf, Germany) and vortexed (Vortex 2 Genie, Scientific Industries, USA) for 30 min at 100 rpm. Sonication was carried out by sonicator Bandelin Sonopuls HD 2070 (Bandelin, Germany) for 30 min. at 4°C and 40 W. Further, mixture was centrifuged at 15,000 g for 30 min (Universal 32 R centrifuge, Hettich-Zentrifugen, Germany).The obtained supernatant was completely evaporated using vacuum evaporator (RVO 400, Ingos, Czech Republic) and subsequently dissolved in 300 µl of acetonitrile. Samples were stored in micro test-tubes at -20 °C at dark prior to the analysis.

2.5 Homogenisation of samples for protein and element determination

Weighed plant tissues (0.2 g) were transferred into a test-tube. Then, liquid nitrogen was added and the samples were frozen to disrupt the cells. Frozen sample was transferred to a mortar and homogenised for 1 min. After it, exactly 1,000 µl of 0.2 M phosphate buffer (pH 7.2) was added and the sample was homogenised for next 5 min. Then, homogenate was transferred into a new test-tube. Frozen samples were subsequently homogenized by shaking on a Vortex–2 Genie (Scientific Industries, USA) for 5 min at 4 °C and sonicated using a Bandelin Sonopuls HD 2070 (Bandelin, Germany) for 10 s at a power of 7 W. Finally, homogenate was centrifuged (14,000 g, 15 min., 4 °C) by a Universal 32 R centrifuge (Hettich-Zentrifugen, Germany). Supernatant was filtered through 0.45 µm Nylon filter discs (Millipore, Billerica, Mass., USA) prior to analysis.

2.6 HPLC-UV measurements

The instrument for HPLC-UV analysis consisted of a solvent delivery pump operating in a range from 0.001 to 9.999 ml.min^{-1} (Model 582 ESA Inc., Chelmsford, MA, USA), and an UV-VIS detector (Model 528, ESA, USA). Sample (20 μl) was injected using an autosampler (Model 542, ESA, USA). Mobile phase consisted of A: acetic acid (50 mM) and B: 100% methanol. Compounds were eluted by linearly increasing gradient: 0 → 6 min (70 % B), 6 → 10 min (100 % B), and 10 → 15 min (100 % B). Detection was carried out at 275 nm. Measurements were carried out at room temperature. Obtained data were processed by the Clarity software (Version 3.0.04.444, Data Apex, Czech Republic). The scheme of the instrument is shown in Fig. 1A.

2.7 HPLC-ED measurements

HPLC-ED system consisted of two solvent delivery pumps operating in the range of 0.001-9.999 ml.min^{-1} (Model 582 ESA Inc., Chelmsford, MA), and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector includes three flow cells (Model 6210, ESA, USA). Each cell consists of four working carbon porous electrodes, each one with auxiliary and dry hydrogen palladium reference electrodes. Both the detector and the reaction coil/column were thermostated. The sample (5 μl) was injected using autosampler (Model 542 HPLC, ESA, USA). Mobile phase consisted of A: acetic acid (50 mM) and B: 100% methanol. Compounds were eluted by linearly increasing gradient: 0 → 6 min (70 % B), 6 → 10 min (100 % B), and 10 → 15 min (100 % B). Detection was carried out at 900 mV. Obtained data were processed by the Clarity software (Version 3.0.04.444, Data Apex, Czech Republic). The scheme of the instrument is shown in Fig. 1A.

2.8 Estimation of detection limit and statistical analyses

The detection limits (3 signal/noise, S/N) and the quantification limits (10 signal/noise, S/N) were calculated according to Long and Winefordner [49], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. RESULTS AND DISCUSSION

PAHs represent group of serious pollutants of the environment with significant impact on animal and human health. In addition, PAHs demonstrate an ability to be accumulated in organisms including plants, which may serve as potential bioaccumulators of these organic pollutants. Due to ability of plants to accumulate organic pollutants, negative effect of plants containing such pollutants on organisms must be carefully considered. In the case of PAHs, benzo[*a*]anthracene, benzo[*a*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a,h*]anthracene and indeno[1,2,3-*cd*]pyrene were identified as potential human carcinogens [50]. Because of all above-mentioned facts, it is necessary to have a robust, rapid and precise analytical technique for

determination of pollutants in complex biological matrices. In this study, HPLC-UV and/or HPLC-ED were optimized for determination of the following PAHs as naphthalene, fluoranthene, pyrene, methylanthracene, triphenylene, benzo[*a*]anthracene, benzo[*a*]pyrene, and coronene.

3.1 Optimization of PAHs separation

Due to high lipophilicity of PAHs, their high retention at the commonly used reverse-phase columns may be expected. Thus, it is advantageous to use chromatographic columns of lower diameter and shortened length. In this case, compounds are eluted more rapidly and, in addition, flow of mobile phase is app. three times lower compared to commonly used columns. We used column Gemini NX (Phenomenex, USA) in this study. The most critical step in separation was represented by separation of fluoranthene and pyrene, which have similar molecular weight and lipophilicity due to similarities in delocalization of π electrons.

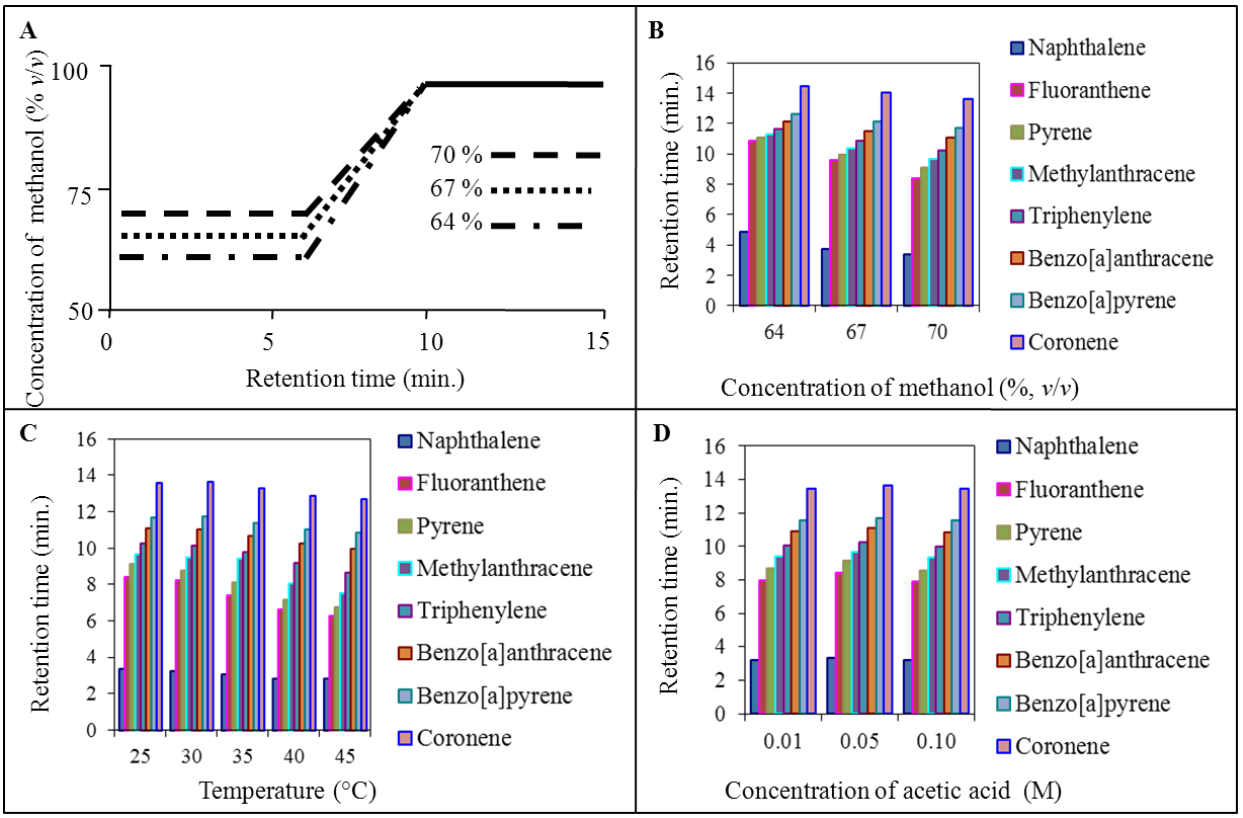

Figure 2. (**A**) Scheme of studied gradients at different concentrations of organic solvent methanol. (**B**) Changes of retention time of studied PAHs according to changes in the content of methanol in mobile phase (64, 67 and 70 % (*v*/*v*)). (**C**) The influence of temperature (25, 30, 35, 40 and 45°C) on retention times of studied PAHs. (**D**) Changes of retention time of studied PAHs according to changes in concentrations of acetic acid (0.01, 0.05 and 0.1 M) in mobile phase.

To optimize HPLC conditions, mixture of PAHs standards (naphthalene 50 µg/ml, fluoranthene 10 µg/ml, pyrene 10 µg/ml, methylanthracene 200 µg/ml, triphenylene 10 µg/ml, benzo[*a*]anthracene 200 µg/ml, benzo[*a*]pyrene 10 µg/ml and coronene 300 µg/ml) was used. Based on our previous

experiments, mobile phase consisted of acetic acid and methanol was used. Considering above-mentioned facts with respect to the separation of fluoranthene and pyrene, isocratic elution from zero to 6th minute was suggested. In addition, the increase of the content of organic solvent methanol to 100 % (*v*/*v*) between 6th and 10th minute with the aim of elution of the most non-polar PAHs benzo[*a*]pyrene and coronene was used, however, starting content of methanol was tested in the mobile phase (64, 67 and 70 % (*v*/*v*)) (Fig. 2A). Changes in methanol content within the range of percents had significant effect on the retention of all separated PAHs. The lowest methanol content in mobile phase (64 %, *v*/*v*) resulted in co-elution of structurally similar PAHs fluoranthene, pyrene and methylanthracene. The medium methanol content (67 %, *v*/*v*) led to the less co-elution of above-mentioned PAHs. The highest methanol content (70 %, *v*/*v*) gave acceptable separation of all PAHs under advantageous time of analysis (Fig. 2B). These changes were observed using both instruments. Further, effects of temperature were investigated. Changes in the temperature usually led to the significant changes in viscosity of a mobile phase containing high content of organic solvents. Viscosity influences also back pressure during separation and retention of separated molecules. For the investigation of effect of temperature on separation, temperatures 25, 30, 35, 40 and 45°C were tested. Retention times of all compounds of interest decreased with the increasing temperature, which was confirmed using both instruments (Fig. 2C). However, this effect negligible i.e. up to 5 % difference between retention times measured at 25 °C compared to retention time measured at 45 °C. Therefore, temperature of 25 °C was used for the following experiments. Concentration of acetic acid as the second part of mobile phase had only minimal effect on the separation of studied PAHs as compounds with high lipophilicity. We studied the effect of three different concentrations of acetic acid as 0.01, 0.05 and 0.1 M in the mobile phase. The medium tested acetic acid concentration (0.05 M) resulted in slightly improved separation of studied PAHs (Fig. 2D). This content was used for the following experiments.

Thanks to the optimization, well separated and symmetric signals of individual PAHs were obtained. Simultaneous determination of all target molecules did not exceed 20 min (Fig. 3A). The retention times of individual PAHs were as follows: 3.20 min (naphthalene), 7.62 min (fluoranthene), 8.28 min (pyrene), 9.26 min (methylanthracene), 10.92 min (triphenylene), 11.02 min (benzo[*a*]anthracene), 11.75 min (benzo[*a*]pyrene), and 14.76 min (coronene) (Fig. 3A). Data introducing regression equations, limits of detection, limits of quantifications, and relative standard deviations for HPLC-UV are summarized in Table 1. The measured calibration curves were strictly linear with regression coefficients higher than 0.991 (Fig. 3B). In the case of HPLC-ED, detection potential was also optimized as one of the most important parameter of the detector. Tested potentials were within the interval from 100 to 900 mV (Fig. 4A). Based on the obtained dependencies it can be concluded that current responses enhanced with the increasing potential. Therefore, we selected this parameter for measuring of calibration dependencies of PAHs of the interest. Separation of the selected PAHs was similar using HPLC-ED and retention times were due to same mobile phase similar to those mentioned above. Nevertheless, detection using electrochemical detector differed markedly in comparison with UV. Linear intervals of the obtained calibration curves measured using HPLC-ED were different for the single PAHs. Calibration curves for pyrene and methylanthracene were linear within the concentration interval from 0.15 to 20 μg/ml (Fig. 4B) and fluoranthene,

benzo[a]anthracene, benzo[a]pyrene, coronene, triphenylene and naphthalene within the concentration interval from 0.78 to 100.00 μg/ml (Fig. 4C). Data introducing regression equations, limits of detection, limits of quantifications, and relative standard deviations for HPLC-UV are summarized in Table 2.

Table 1.HPLC-UV analytical parameters of polycyclic aromatic hydrocarbon simultaneous determination.

Compound[a]	RT[b] (min.)	Regression equation	Linear dynamic range(μM)	Linear dynamic range(μg/ml)	R^2	LOD[c] (μM)	LOD (ng/ml)	LOD (fmol/injection)	LOQ[d] (μM)	LOQ (μg/ml)	LOQ (fmol/injection)	RSD[e] (%)
Naphthalene	3.20	y = 0.2597x - 0.1281	6.085 - 780.21	0.78 - 100	0.9987	6	0.8	130	22	3	430	4.6
Fluoranthene	7.62	y = 0.6332x + 0.1410	1.928 - 494.41	0.39 - 100	0.996	2	0.3	40	6	1	110	3.8
Pyrene	8.28	y = 0.7015x - 0.0280	1.928 - 494.44	0.39 - 100	0.9997	2	0.3	30	5	1	100	3.6
Methylanthracene	9.26	y = 0.0472x - 0.0419	6.833 - 438.04	1.56 - 100	0.9978	20	5.0	400	67	15	1300	4.3
Triphenylene	10.92	y = 1.0838x + 0.0662	0.210 - 438.04	0.39 - 100	0.9997	1	0.2	20	3	1	60	3.9
Benzo[*a*]anthracene	11.02	y = 0.0343x - 0.0022	8.114 - 520.13	0.39 - 100	0.9976	30	6.0	660	109	21	2200	4.1
Benzo[*a*]pyrene	11.75	y = 0.6105x - 0.0099	0.753 - 396.34	1.56 - 100	0.9979	1	0.4	30	5	1	90	4.8
Coronene	14.76	y = 0.0425x - 0.3392	41.616 - 332.93	0.39 - 100	0.9911	20	5.0	340	56	17	1100	4.5

a…studied PAHs
b…retention time
c…limit of detection (3 S/N)
d…limit of quantification (10 S/N)
e…relative standard deviation (n=3)

Table 2.HPLC-ED analytical parameters of polycyclic aromatic hydrocarbon simultaneous determination.

Compound[a]	RT[b] (min.)	Regression equation	Linear dynamic range (μM)	Linear dynamic range (μg/ml)	R^2	LOD[c] (μM)	LOD (ng/ml)	LOD (fmol/injection)	LOQ[d] (μM)	LOQ (μg/ml)	LOQ (fmol/injection)	RSD[e] (%)
Naphthalene	3.20	y = 2.142x + 59.451	12.17 - 780.21	1.56 - 100	0.8913	5	0.7	55	18	2	182	3.6
Fluoranthene	7.62	y = 5.3723x + 158.68	3.86 - 494.41	0.78 - 100	0.9918	1.5	0.3	14	5	1	46	4.2
Pyrene	8.28	y = 87.141x - 58.636	0.77 - 98.89	1.56 - 20	0.997	0.1	0.02	1	0.3	0.06	3	3.1
Methylanthracene	9.26	y = 66.973x + 133.32	0.68 - 87.61	1.56 - 20	0.9901	0.1	0.02	1	0.3	0.08	3	2.6
Triphenylene	10.92	y = 1.4362x + 12.194	13.69 - 438.04	3.13 - 100	0.9599	4.6	1	46	15	3	152	3.9
Benzo[*a*]anthracene	11.02	y = 1.5377x + 108.62	4.06 - 520.13	0.78 - 100	0.9976	5	1	51	17	3	169	3.5
Benzo[*a*]pyrene	11.75	y = 5.8942x + 15.929	3.09 - 396.34	0.78 - 100	0.9916	1	0.3	10	3	1	34	4.9
Coronene	14.76	y = 1.3876x + 76.905	2.60 - 332.93	0.78 - 100	0.9909	4	1	36	12	4	120	3.2

a…studied PAHs
b…retention time
c…limit of detection (3 S/N)
d…limit of quantification (10 S/N)
e…relative standard deviation (n=3)

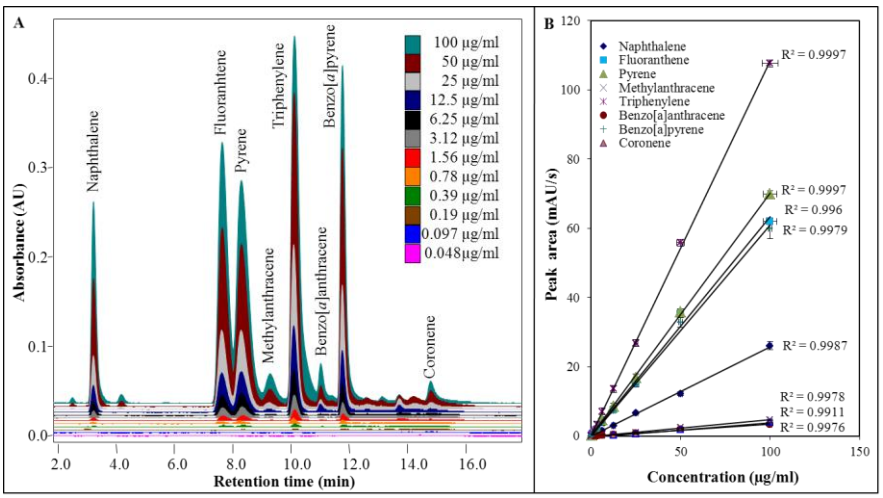

Figure 3. (**A**) Overlay of HPLC-UV chromatograms of simultaneously detected PAHs naphthalene, fluoranthene, pyrene, methylanthracene, triphenylene, benzo[*a*]anthracene, benzo[*a*]pyrene and coronene in a mixture. (**B**) Calibration curves with calibration equations and regression coefficients for individual PAHs. Experimental conditions were as follows: Mobile phase - A: acetic acid (50 mM) and B: methanol (100 %). Compounds were eluted by linearly increasing gradient: 0→6 min (70% B), 6 → 10 min (100 %B), 10 → 13 min (100 % B), 13 → 14 min (70 % B), 14 → 20 min (70 % B). Detection was performed at 275 nm. Time of one analysis was 20 minutes.

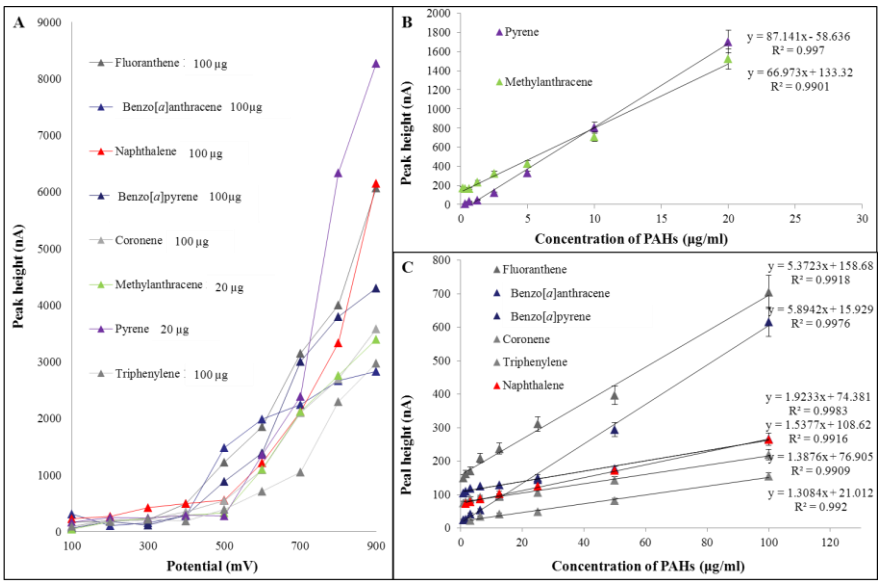

Figure 4. (**A**) Dependences of peak heights of naphthalene, fluoranthene, pyrene, methylanthracene, triphenylene, benzo[*a*]anthracene, benzo[*a*]pyrene and coronene on working electrode potential. Calibration curves for (**B**) pyrene and methylanthracene measured within the concentration interval from 0.15 to 20 µg/ml and (**D**) fluoranthene, benzo[a]antracene, benzo[a]pyrene, coronene, triphenylene and naphthalene measured within the concentration interval from 0.78 to 100.00 µg/ml. Experimental conditions were as follows: Mobile phase - A: acetic acid (50 mM) and B: methanol (100 %). Compounds were eluted by linearly increasing gradient: 0→6 min (70% B), 6 → 10 min (100 %B), 10 → 13 min (100 % B), 13 → 14 min (70 % B), 14 → 20 min (70 % B). Detection was performed at 900 mV (Figures B and C). Time of one analysis was 20 minutes.

3.2 Analysis of real samples

Optimization of HPLC technique for determination of above-mentioned polycyclic aromatic hydrocarbons brings new possibilities in their analysis, especially due to shortening of the time of analysis and improvement of the sensitivity. There are numerous papers focused on PAHs determination. However, analytical techniques must be modified in accordance with character of biological matrix, which are chemically and physically very different from gaseous samples, through waters and to organic matters. Rey-Salgueiro et al. focused on HPLC determination of different PAHs including their hydroxyl derivatives in different biological samples [51,52]. Compared to our equipment, authors used fluorescence detector, which is not routinely used in connection with the HPLC technique. In comparison with commonly used techniques used in PAHs analysis, such as gas chromatography with flame ionisation detector or mass spectrometry detector, and electrochemical techniques, laser-induced fluorescence technique is used for accurate analysis of PAHs in liquids [53,54]. However, HPLC technique represents "gold" standard in analysis and, in addition, belongs to the common analytical tool in laboratories. Because accessible, rapid and precise PAHs analysis is required, HPLC technique plays still crucial role in their analysis. Therefore, our method can be used for analysis of real samples with sufficient sensitivity and repeatability.

To test the applicability of our method, pea plants (*Pisum sativum* L. cv. Zazrak) were treated with fluoranthene (FLT) in the concentrations 0 (two control groups, both non-treated with fluoranthene but one treated with DMSO as solvent used for preparing of stock solutions of the target molecule), 0.1, 1.0 and 5.0 mg/l and their tissues analysed to determine applied PAHs. Pea plants were chosen due to their agricultural importance as a farm crop, thus, they can play important role in food chain. In the first step, extracts from individual plant tissues as root, stem and leaves were prepared. It has been shown that results of analyses significantly depend on the way of preparation of real samples including usage of solvent or mixture of solvents and storage of samples. Especially storage of samples (exposition to UV, effect of temperature) is connected with chemical modification of PAHs. Dimethyl sulphoxide, benzene, acetonitrile, hexane, methanol and dimethyl chloride and their mixtures are commonly used as the most suitable solvents for PAHs extraction. Due to only low levels of some PAHs in biological matrixes, pre-concentration and purification of the samples of interest by using of solid phase extraction, liquid-liquid or solid phase extractions, micro-extraction techniques immunosorbents, column adsorption chromatography, high performance liquid chromatography must be carefully considered.

Purification of samples is in many cases unavoidable. In our case, choice of solvent enabling us to extract fluoranthene from plant tissues without some labour pre-concentration steps was the most important.

Firstly, plants were divided into individual plant tissues as leaves, stems and roots, which were used for preparation of extracts for analysis. These extracts were used also for the determination of protein content and content of calcium, iron, copper, and zinc ions. Finally, both changes in FLT distribution and protein and ions content of treated pea plants were determined. The obtained results are discussed in separate sub-chapters.

3.3 Determination of proteins content

Treatment of pea plants by FLT led to significant changes in protein content in the individual leaves, stems, and roots. The highest protein content was found in stems of control plants followed by roots and leaves. In the pea plants treated with the highest concentration of FLT (5 mg/l), total protein content in individual organs decreased in the following order leaves > roots > stems. The total protein content was reduced almost for 30 % in comparison with control untreated plants. Generally, total protein content was lowered in all FLT concentrations except of stems of plants treated with the lowest FLT concentration (0.1 mg/l). In this case, total protein content was very slightly increased compared to control (Fig. 5A).

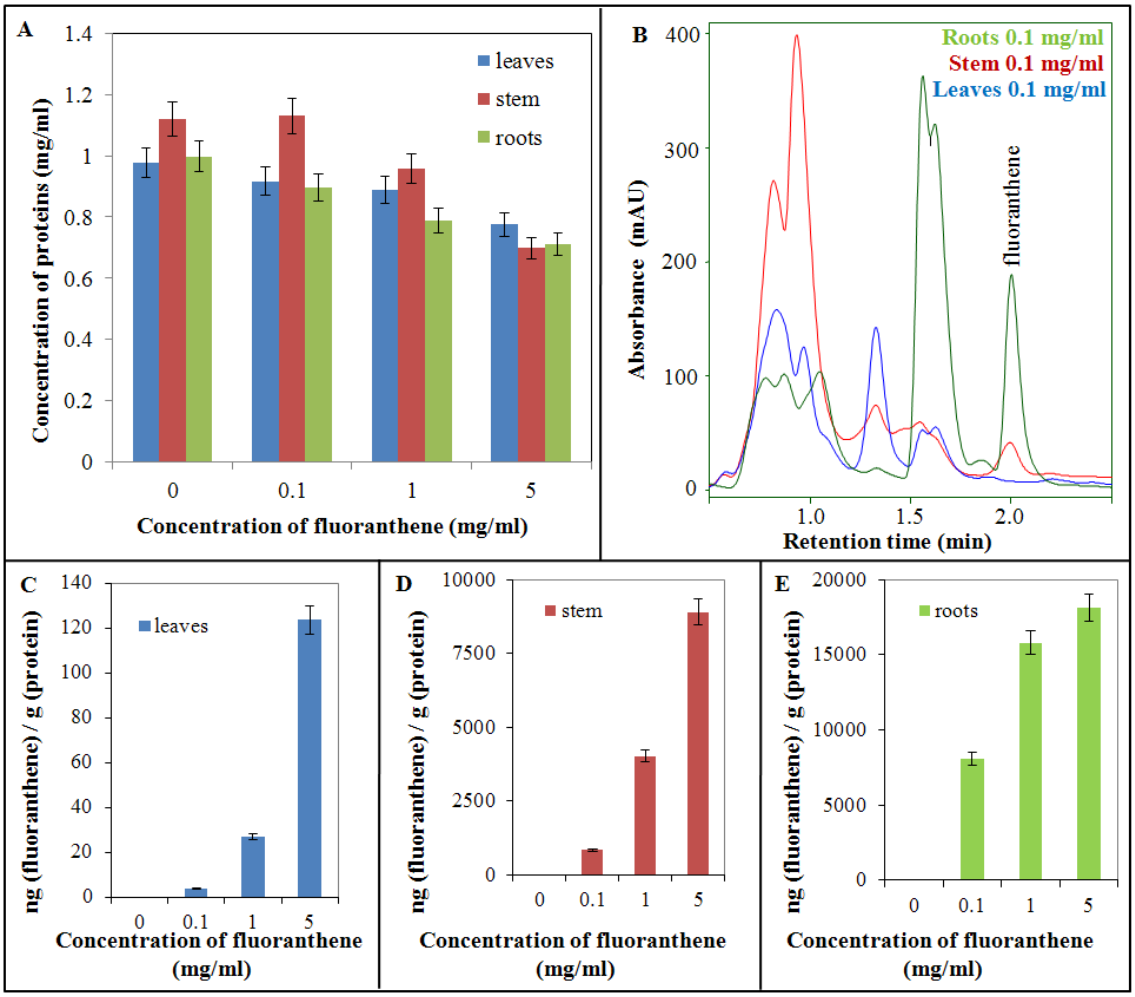

Figure 5. (**A**) Overlay of chromatograms of analysed pea plants treated with FLT in concentration of 0.1 mg/ml. (**B**) Content of total proteins in roots, stems and leaves. Content of FLT in (**C**) roots, (**D**) stems and (**E**) leaves of treated plants.

Proteins play essential role in control and regulation of plant growth and development, they carry out special roles connected with detoxification of xenobiotics. Effect of PAHs on proteosynthesis stays still unknown. Some papers are focused on studying an effect of FLT on weight of experimental animals. Reduction of animal weight and protein biosynthesis reduction is demonstrated in the paper of

Knuckles et al., who tested effect of FLT on F-344 rats [55,56]. Induction of cytochrome (CYP), which is connected with variety of cellular processes including xenobiotics metabolizing, fatty acid metabolism and bile acid biosynthesis, was determined in *Fundulus heteroclictus*, fingerling fish, after exposition to benzo[*a*]pyrene [57]. Similar effects of PAHs on CYPs were determined in annelids [58]. In studies on bacteria, which are partially able of PAHs biodegradation, changes in protein profiles have been shown too. *Ochrobactrum anthropi* strain BAP5, which is able to metabolize benzo[*a*]pyrene, demonstrated changes in some dehydrogenases, lyases, oxidative cyclases and ABC transporters after benzo[*a*]pyrene exposition [59,60]. There is very few papers describing changes of protein profile in plants after PAHs exposition. Only one study on *Arabidopsis thaliana* cultivated in vitro demonstrates the effect of phenanthrene on oxidative stress [61]. This study is focused on determination of enzyme activities connected with oxidative stress as superoxide dismutase (SOD), peroxidase (PX), catalase (CAT), ascorbate peroxidase (APX), and glutathione. In conclusion, data focused on effect of PAHs on protein biosynthesis in plants are still missing.

3.4 FLT transport within a plant

In our following experiments, we aimed at using of our above-optimized method for determination of FLT in plant extracts. HPLC-ED chromatogram of real sample is shown in Fig. 5B, It can be concluded that FLT demonstrated mobility within plant body. However, the highest FLT levels were detected in roots, which were directly exposed to FLT. This fact indicates ability of FLT to bind into different tissues of roots. Due to FLT lipophilicity, we can assume its accumulation in lipophilic cellular compartments such as plasma membrane and membrane-based organelles. The unanswered question consists in possible chemical modification of FLT with subsequent interactions with numerous hydroxyl groups of polysaccharides of cell walls as cellulose, hemicelluloses and pectin substances, which may be responsible for FLT immobilization and accumulation. In comparison with roots, FLT content was two-time lower in stems and almost 150-time lower in leaves in average. These results indicate on accumulation of FLT in root tissues and on only limited transport into aerial parts. This is very well shown in Figs. 5C, D and E, where the FLT content in different plant parts. Based on the results it can be concluded that content of FLT markedly decreased from roots to leaves. There is two-fold difference. Moreover, there is increasing trend in the content of FLT in plant parts with the increasing concentration of the selected PAHs. On the other hand, we were focused on the determination of only fluoranthene, thus, formation of more reactive FLT derivatives with hydroxyl groups may play crucial role in toxicity. It has been shown that PAHs are metabolized into complicated group of quinones, phenols, dihydrodiols and thiols in animals. Especially introduction of hydroxyl group/groups represents crucial step in interactions with biomolecules, such as DNA or proteins with possible induction of pathological processes [62]. However, analysis of FLT metabolites in plants is practically impossible due to possible radical way of FLT modification and formation of reactive radical FLT forms, such as epoxides, which have due to their reactivity only very short lifetime. In addition, determination of FLT in plant tissues is complicated by possible incorporation of modified FLT into polysaccharides and lignin, where rigid bindings can be supposed. These chemical

modifications may be caused by activity of peroxidases and oxidases, which are crucial in the process of lignification. PAHs uptake by plants has been demonstrated in some papers; however detailed studies are still missing. It is well known that plants are able to uptake these class of compounds via root system. This way of uptake is complicated due to low solubility of PAHs in water. In the case of PAHs uptake via aerial parts, they are deposited in lipophilic substances on the plant surface (cuticle, waxy layer), or they are able to entry the plants via stomata as integral part of gas [30,63]. Uptake of PAHs by roots is modified by many parameters including soil composition, presence of organic acids and higher organic compounds, possibility of modification of PAHs structure, especially by UV radiation (exposition to the solar radiation), but also by the presence of microorganisms, such as bacteria and fungi in soil, which can enzymatically modify PAHs structure and physico-chemical properties.

3.5 Metal ions determination

Despite the fact that data about effect of fluoranthene on transport of nutrients including minerals from soil are still missing, effect of FLT on their uptake can be supposed, especially with respect to ability of fluoranthene to interact with biomembranes under disruption of their function [64]. Homeostasis of metals ions is strictly maintained and controlled by peptides and proteins rich in –SH moieties e.g. reduced glutathione or phytochelatins, which are responsible for their translocation and deposition in cellular compartments [65-78]. These proteins participate also in detoxification of xenobiotics, thus, mutual interactions in uptake of minerals and FLT are possible, but still unknown. In some papers, changes in uptake of some elements due to PAHs treatment have been demonstrated. FLT and minerals uptake is connected especially with activity of soil bacteria and fungi [79]. Some soil microorganisms are able to degrade PAHs with resulting modification of their uptake by plants [80,81]. In the group of fungi, sooty moulds, dark pigmented non-parasitic fungi superficial on plants, respectively plant roots, were identified as possible PAHs modifiers. Due to production of some enzymes such as laccase, they are able to modify PAHs uptake by plants [21,82]. However, there is only little knowledge about effect of PAHs on uptake of mineral nutrition from soil. Ecologically focused papers demonstrated an effect of PAHs on heavy metals ions uptake by plants [83,84]. It has been demonstrated that some metal ions (such as iron(III)) are able to enhance PAHs chemical modification and biodegradation in soils [85].

FLT treatment led to increase of calcium(II) ions levels in roots and stems (0.1 mg/l), however, in the middle applied FLT concentration (1.0 mg/l) calcium(II) ions content was markedly increased in leaves, stems and roots in comparison with control plants. In the highest FLT concentration (5 mg/l), calcium(II) ions contents were decreased in all organs except roots in comparison with control plants (Fig. 6A). The role of calcium ions in plants is well known, nevertheless, connection between their levels and oxidative stress is practically unknown. Some papers demonstrate their crucial signalling role in oxidative stress. In addition, involvement of calcium ions in reduce/oxidized glutathione (GSH/GSSG) homeostasis was demonstrated in the paper of Rentel et al. [86] and Liu et al. [80].

However, connection between calcium ions and oxidative stress caused by PAHs must be further investigated.

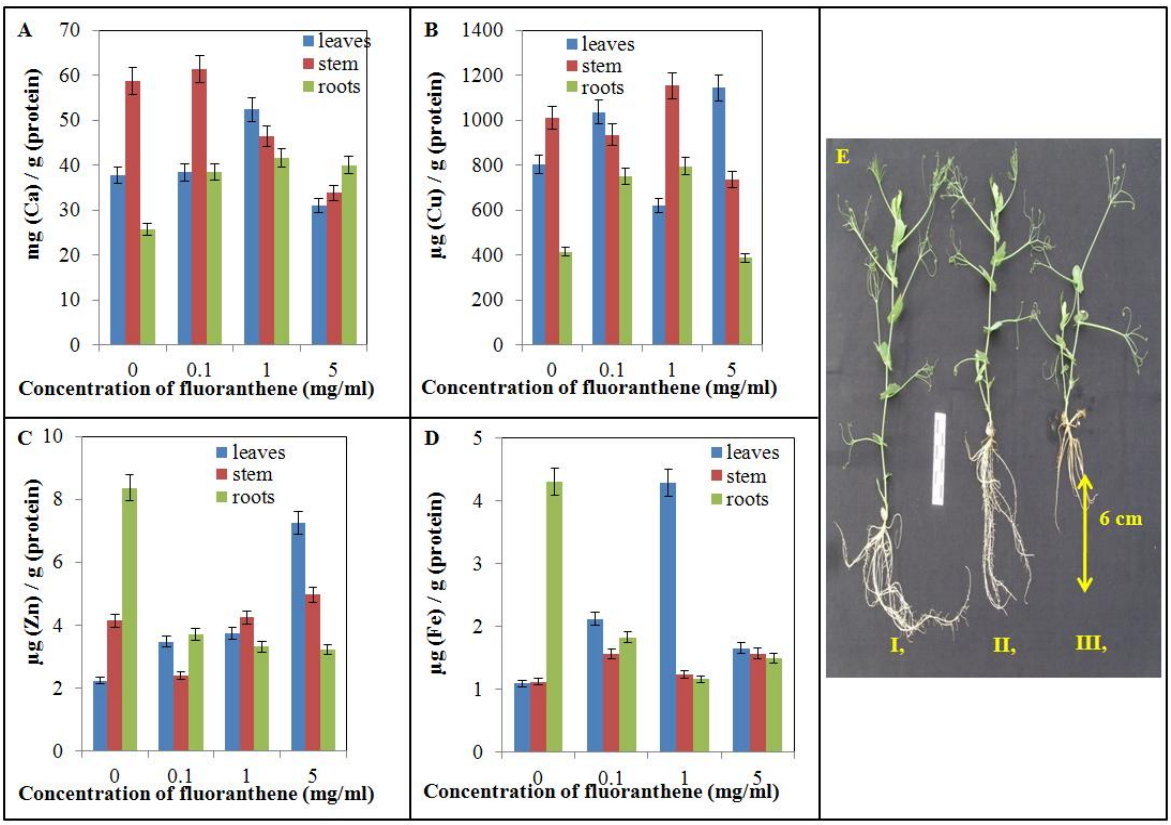

Figure 6. Content of (**A**) Ca, (**B**) Cu, (C) Zn and (**D**) Fe ions in roots, stems and leaves of control and experimental pea plants treated by FLT. (**E**) Photograph of pea plants in eighteenth day of treatment after FLT exposition in concentrations 0.1 (I), 1.0 (II) and 5.0 (III) mg/ml. Changes in growth, especially in formation of root system (formation of lateral roots), are well visible.

Unambiguous results were obtained by determination of copper(II) ions, which are connected with some detoxification enzymes, such as superoxide dismutase (SOD). FLT treatment led almost in all concentrations to reduction of copper(II) ions level in roots and stems compared to control plants (Fig. 6B). Copper as important micronutrient plays crucial role in plant growth and development. Copper ions represent essential co-factors of some metalloproteins, which are necessary for electron transport in photosynthesis, mitochondrial respiration, oxidative stress responses, biosynthesis of cell wall and hormone signalling [87-90]. In addition, copper(II) ions represent co-factors of many enzymes including Cu/Zn superoxide dismutase, cytochrome c oxidase, amino oxidases, laccase, polyphenol and plastocyanin oxidases [89]. Decrease of detectable copper ions because of FLT treatment can be connected directly with Cu/Zn SOD, whose enhancement under PAHs treatment has been demonstrated in the paper of Liu et al. [61].

Zinc(II) ions levels were increased in leaves of all experimental variants. The most distinct increase was found in the highest applied FLT concentrations (Fig. 6C). Decrease of zinc(II) ions

content related to control was determined in roots of plants of all experimental variants with maximal decrease in plants treated with the highest FLT concentration. In stems, decrease of zinc(II) ions level in the lowest FLT concentration (0.1 mg/l) followed by its enhancement in all other FLT concentrations was well evident. Zinc(II) ions as integral part of zinc fingers, which play crucial role activation of genes transcription, may serve on molecular-biological level in regulation of transcription, especially in transcription of proteins connected with detoxification [91,92]. These findings correspond to the levels of total protein, thus, association between zinc ions and proteosynthesis should be considered.

The highest iron(II) level was determined in roots of control plants (Fig. 6D). FLT treatment led to reduction of iron(II) content in roots of all experimental variants. Contrariwise, iron(II) content was markedly enhanced in stems and leaves in all experimental variants. Level of iron(II) ions in leaves of pea plants treated with FLT in concentration of 1.0 $mg.l^{-1}$ was comparable to its content in roots of control plants. Decrease of iron(II) content in roots may be connected with protective activity of some phenolic compounds. It has been demonstrated that iron(II) ions are able to chelate polyphenol compounds, such as flavonoids. Therefore, it is possible that free iron(II) ions form complexes with polyphenols in roots, which have significant antioxidant activity and play role in protection against oxidative stress, and contribute to enhanced oxidative stress in roots. In addition, iron ions are donors and acceptors of electrons and they are important not only in the processes of photosynthesis and respiration, such as sugar metabolism [93-95], but also in Fenton´s reaction [96]. Due to fact that Fe^{2+} ions can interact with hydrogen peroxide under formation of hydroxyl radicals, role of iron in oxidative stress caused by PAHs exposure must be further investigated.

Analysis of samples of cultivation media demonstrated that FLT treatment leads to changes in uptake of above-discussed elements. In the lowest FLT concentration, uptake of all elements (Ca, Cu, Zn and Fe) was increased, however, in the highest FLT concentration, Ca, Cu, Zn and Fe uptake was markedly reduced in comparison with control plants. This fact must be considered in connection with FLT accumulation in roots and changes in apoplastic and symplastic way of transport of water and minerals. Moreover, morphological changes of pea plants after FLT treatment included especially i. reduction of root system, ii. changes in formation of lateral roots, and iii. changes in leaf area and are shown in Fig. 6E.

4. CONCLUSIONS

Hydroponically cultivated pea plants treated with polycyclic aromatic hydrocarbon fluoranthene in concentration range from 0 to 5 mg/l demonstrated its accumulation in roots, however, its translocation within plant body was only limited. Marked changes were detected in total protein levels and levels of elements, which play essential role both in processes of plant growth and development and in responses of plants to oxidative stress. However, all these changes must be further investigated, especially in association with oxidative stress and disruption of root function in connection with FLT accumulation. Our study represents focus on basic knowledge about protein biosynthesis and levels of important ions – calcium, copper, zinc and iron in plants treated with PAHs.

Our results indicate possible participation of these metal ions in stress response to fluoranthene exposure; nevertheless, other experiments focused on mobilisation of these ions as well as on protein biosynthesis and involvement of PAHs in these processes should be carried out to get more information about effect of PAHs on plants.

ACKNOWLEDGEMENTS
The financial support from GA CR 522/09/0239, MSMT 6215712402 and CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged. The results were presented at 11th Workshop of Physical Chemists end Electrochemists held in Brno, Czech Republic.

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