Investigation on Parabens Occurrence in Romanian WWTP Sludge by LC-MS/MS Method

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A sensitive and precise method for detection of five parabens in sewage sludge was developed based on ultrasonic assistedextraction, SPE clean-up and LC-MS/MS. Most of the parameters that affect the extraction step such as type of solvent and volume, extraction time were optimized. For ultrasonic extraction it was used a mixture of methanol: acetone, then the extract was centrifuged in order to obtain clean supernatant and the extract was purified on polymericSPE cartridges. The parabens were separated on C18 column in 13 min at 18ºC using a gradient of mobile phase of 0.01% acetic acid and acetonitrile. Using the method, limit of quantitation (LOQ) were obtained ranging from 0.4 to 2ng/g. All recoveries ranged from 71% to 109% for all compounds. The repeatability and reproducibility between days expressed as RSD (%) were less than 7.4% and 14.5%, respectively. The sum concentrations of all parabens for each sludge sample ranged between 19 ng/g dry weight and 32.7 ng/g dry weight. The study of the profile sample composition shows that the average contribution of each compound of the total parabens concentration was as follow: methyl paraben 62.9%, ethyl paraben 18.6%, iso-propyl paraben 10.9%, propyl paraben 10.8%. This result indicate that methyl paraben is the most used paraben followed by ethyl paraben.

Keywords: parabens, liquid chromatography-mass spectrometry, sludge, ultrasonic extraction

Alkyl esters of p-hydroxybenzoic acid, also named parabens, are intensively used as antimicrobial agents in food products, and preservatives in pharmaceutical preparations, in personal care products (cosmetic and toiletries consumer's products) because of their broad antimicrobial spectra, good stability over a high *p*H range, moderate solubility and non-volatility [1, 2]. Endocrine-disrupting compounds represent a group of organic contaminants, including natural substances (e.g., phytoestrogens) and synthetic compounds like polychlorinated biphenyls, polybrominated biphenyls, dioxins, bisphenol A, alkyl phenols, parabens, pesticides, fungicides, phthalates, and pharmaceutical agents (e.g., diethylstilbestrol and tamoxifen) that may interfere with the endocrine system [3, 4]. Their antimicrobial activity increases when increasing ramification of the esteric chain. These compounds have estrogenic activity and are potential toxic for some aquatic organisms like fish and intervertebrates [5, 6].These compounds are emerging endocrine disruptors that give immune dysfunction and affect human reproductive capabilities [7]. Some researchers propose a connection between parabens and the risk of breast cancer, so methyl paraben was detected in human breast tumors in concentration of 12.8 ng/g [8]. Due to this problem, companies are trying to produce products free of paraben. The most used parabens in commercial products include methyl paraben, ethyl paraben, propyl paraben, butyl paraben and benzyl paraben. It is known that parabens are continuously discharged in the environment by effluents of waste water treatment plants and by sewage sludge [9]. In the USA a study reported parabens detection in sludge in concentrations which ranged between 0.5 ng/g propyl paraben and 15.9ng/g methyl paraben [7]. In Spain other paper reported parabens determination in sludge at concentration of 26ng/g methyl paraben, 44.1ng/g propyl paraben [10]. For extraction of organic analytes, including parabens, from solid samples, were reported extraction methods such as pressurized liquid extraction (PLE), ultrasonic assisted extraction (UAE), supercritical fluid extraction (SFE) and for their analysis is done using gas chromatography-mass spectrometry (GC-MS/MS), liquid chromatography coupled with mass spectrometry (LC-MS/MS) [7, 10-14].Detection of the parabens in sludge is important due to their potential risk to human and animal health. Parabens perturb the endocrine system, generate estrogenic activity, can produce cancer, and affect reproductive system. It is known that treated sewage sludge is applied in many countries on the agricultural land and this procedure can generate pollution of soil, ground water and surface water. Parabens removal rates from WWTP, mainly in aerobic systems, are high. Degradation is correlated with the length of parabens carbon chain. Short chain compounds, such as methyl paraben and ethyl paraben, can be 99% broken down in 2.1 days. But then, propyl paraben and butyl paraben can be degraded in proportion of 99% in 3.7 and 4.5 days [5]. In Romania the scientists mainly developed chromatographic methods for detection of organic compounds in liquid environment matrices such as drinking water, ground water, waste water and surface water [15-19] and indoor/ambient air [20, 21]. There is insufficient information about presence of parabens in environment, their fate and potential toxic effects on living organisms. The limited environmental information's about the presence of parabens in waste water treatment plant (WWTP) sludge represent an important research issue. Thus, this study was performed to obtain the first Romanian research regarding parabens occurrence in municipal WWTP sludge. Such analytical investigation provides important baseline concentration data for the assessment of potential environmental effects from exposure to parabens in soil. The objective of this study was to develop a new and sensitive method for identification and quantification of

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some parabens using ultrasonic extraction followed by LC-MS/MS and to study their occurrence in sewage sludge samples from urban WWTP. The internal standard method, using an appropriate labelled internal standard (IS), is considered to be one of the best methods to compensate for matrix effects. By adding IS to the sample before its treatment, the potential analytical errors associated to the sample manipulation can be also compensated.

Experimental part

Reagents and standards

Methyl-paraben (MPB), ethyl-paraben (EPB), propyl paraben(PPB), isopropyl paraben (IPB), benzyl paraben (BPB) (mix 10mg/L in acetone), and isotopically labeled ethyl 4-hydroxybenzoate-ring-$^{13}C_6$ (EPB13C6, 10mg/L in acetone) used as surrogate internal standard for the quantitation of the targeted analytes were obtained from Sigma Aldrich (Steinheim, Germany). Initially we prepared an intermediary standard solution containing 500µg/L mixed parabens in acetonitrile. After that six calibration solutions (in mobile phase) in the range 1-100 µg/L were obtained by successive dilutions from a 500µg/L mixed parabens intermediary standard solutions. Each calibration solution contained 50µg/L ethyl 4-hydroxybenzoate-ring-$^{13}C_6$ (internal standard). Stock standard solutions of compounds were stored at -20°C. LC grade acetonitrile (ACN), methanol (MeOH), acetone, acetic acid (p.a.), formic acid (p.a.), were acquired from Merck (Darmstadt, Germany). The ultrapure water was obtained with a Milli-Q water purification system (Milipore Bedford, MA, USA). The Strata X (500 mg, 6 mL) cartridges used for solid phase extraction were purchased from Phenomenex (Torrance, CA, USA).

Sample preparation

The sewage sludge samples were collected manually by a conveyor belt subsequent to dewatering from a municipal WWTP located in southeastern Romania. The over 24h collected samples were composite samples of sludge and were kept on ice during transit to the laboratory where they were stored at -20°C until treatment took place. A mass of 100g of sludge was collected in a brown glass jar. All samples were collected in the periods 7-11 December 2017 for 5 days consecutively every time. Sludge samples were first lyophilized in a Christ Alpha 1-2 LD lyophilizer (Martin Christ GmbH, Germany) for 24 h, crushed and homogenized by a mortar and pestle, sieved (particle size <100µm) and they were stored in glass bottles at -20°C until they were analyzed. A lyophilized sludge sample (0.5g) was weighed into a glass baker and then 1mL of 50 µg/L internal standard was added. Sludge samples were introduced into centrifuge tubes where 5 mL of a mixture of methanol and acetone (1:1, v/v) was then added. The obtained mixtures were placed in an ultrasonic bath (Bandelin, Sonorex) for 15 min, after which they were centrifuged for 10 min at 3000 rpm. The supernatants obtained were transferred with a pipette to an extraction vial. The extraction process was repeated by adding 5 mL of the above solvent mixture and the obtained extracts were combined and then diluted with 100 mL ultrapure water. Thus, the methanol content was decreased below 10% and it was eliminated his contribution to analytes elution from SPE cartridge. After that the diluted extract was purified on Strata X cartridges (500 mg, 6 mL) using the Auto Trace 280 (Thermo Scientific) automated solid phase extraction (SPE) system. Each cartridge was conditioned with 10 mL of methanol followed by 10 mL of ultrapure water at a flow rate of 10 mL/min. After the entire amount of sample passed through the cartridge at a flow rate of 10 mL/min, the adsorbent phase was washed with 20 mL of ultrapure water at a flow rate of 10 mL/min. The adsorbent phase was dried under vacuum for 25 min. Target analytes were eluted using a volume of 2 x 5 mL methanol in a concentration tube at a flow rate of 10 mL/min. The eluates were dried under a gentle stream of nitrogen in a water bath at 40 ± 5°C, then reconstituted in 1mL of mobile phase (acetonitrile: water with 0.01% acid acetic) and transferred to a vial for chromatographic analysis. Solutions extracts were filtered through a 0.45µm Millipore filter (cellulose) before being introduced into the vial.

LC-ESI-MS instrument and parameters

Determination of parabens was performed with an Agilent 1260 series UHPLC system (Agilent, Waldbronn, Germany), which was constituted of a column thermostat, autosampler and a binary pump coupled with an Agilent 6410B triple quadrupole mass spectrometer with electro-spray ionization (ESI). Data acquisition and analysis were performed using Mass Hunter software, revision B.04.01. The chromatographic column was Hypersil Gold C18 (2.1x100mm, 3µm) from Thermo Scientific and the injection volume was 10µL. The mobile phase rate (composed by acetonitrile and 0.01% acid acetic) was 0.2mL/min and the column temperature were kept at 18°C. For the separation of analytes, we used a mobile phase

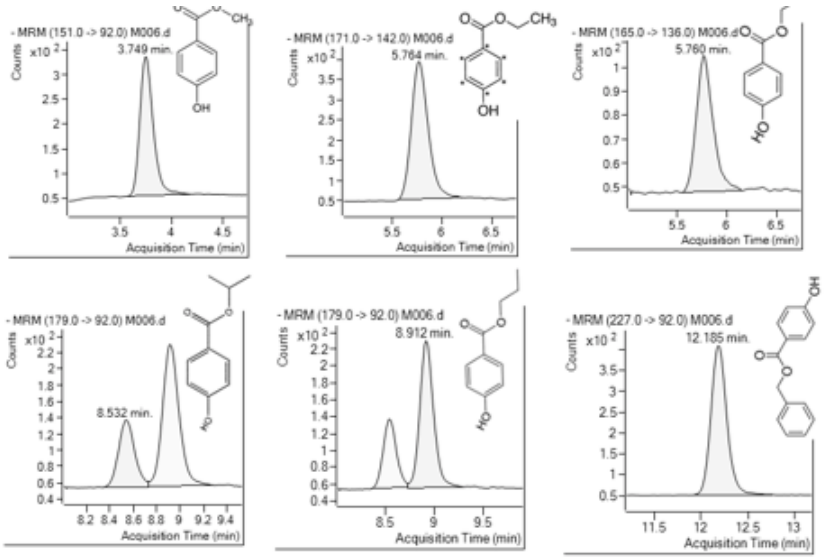

Fig.1. MRM transitions obtained for a standard solution containing 10µg/L mixed parabens in mobile phaseand the internal standard (IS) 50µg/L, from left to right: MPB, EPB, EPB-ring13C6 (IS), IPB, PB, BPB

Table 1
GRADIENT ELUTION PROGRAM USED TO SEPARATE THE FIVE PARABENS

Time (min)	Acetonitrile (%)	Acetic acid (%)
0.00	30	70
3.00	42.5	57.5
6.00	42.5	57.5
12.00	68.5	31.5
12.10	30	70
13.5	30	70

Compound	Time segment (min)	Retention time	MRM Transition	Fragmentor voltage (V)	Collision energy (V)	Dwell time (msec)
MPB	0-5	3.72	151→136 151→92	80 80	5 15	200 200
EPB-$^{13}C_6$	5-8	5.73	171→142 171→98	100 100	15 5	100 100
EPB	5-8	5.73	165→136 165→92	100 100	15 5	125 125
IPB	8-11	8.5	179→136 179→92	100 100	5 15	200 200
PPB	8-11	8.89	179→136 179→92	100 100	5 15	200 200
BPB	11-15	12.17	227→136 227→92	90 90	5 15	200 200

Table 2
MRM TRANSITIONS AND MS/MS OPERATING PARAMETERS SELECTED FOR THE ANALYSIS OF TARGET PARABENS

gradient elution as can be seen in table 1. A post run time was set at 8.5 min for column equilibration.

The MS optimum negative ionization parameters were: gas temperature - 300°C, gas flow -6L/min, capillary voltage - 4000V, nebulizer pressure - 40psi. The MS acquisition of signals was performed in Multiple Reaction Monitoring (MRM). Retention times, MRM transitions, collision energies, fragmentor voltages, and other MS parameters are presented in table 2. For each compound, two fragments of the de-protonated molecule $[M-H]^-$ in negative ionization mode were monitored. Two MRM transitions were used, the most intense/abundant for quantitation (Quantifier) and the second most abundant for confirmation (qualifier). In figure 1 is presented the MRM chromatogram of extracted ions obtained for a standard solution containing 10 µg/L mixed parabens in mobile phase and the internal standard 50 µg/L.

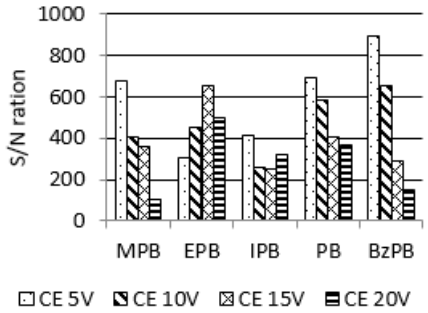

Fig.2a. S/N ratio variation with collision energy during MS method optimization for parabens

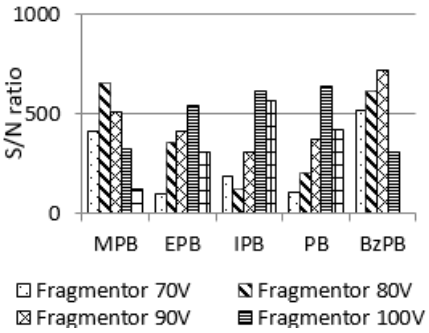

Fig. 2b. S/N ratio variation with fragmentor voltage during MS method optimization

Results and discussions

LC-MS/MS parameters optimization

The main parameters that affect the chromatographic separation (flow rate, column temperature) were studied. The best separation, peak shape, intensity of signal and retention time were obtained with 0.2mL/min flow rate and 18°C. Mass spectrometric detection parameters were optimized to obtain highest possible sensitivity when working in MRM mode. The effect of mobile phase on ionization of parabens was studied. Mobile phase is an important factor in LC-MS/MS analysis. Mobile phase components A of different composition (0.01-0.1% acetic acid, 0.01-0.1% formic acid) and different mobile phase components B (methanol and acetonitrile) were tested for optimization of the LC mobile phase. The formic acid showed higher background noise and lower S/N ratio than acetic acid and he was disregarded. It was observed that decreasing acetic acid concentration from 0.1 to 0.01%, generated a significant increase in S/N ratio for all parabens. The increase may be generated by higher ionization of these compounds in the presence of lower acetic acid concentration. Further decrease of acetic acid to 0.001% generated a decrease in sensitivity for all analytes. Due to the acidic phenol groups in their molecule, negative ESI ionization proved to be more sensitive than positive with less ionization suppression, due to complex matrix. Among the two organic eluents, methanol generated poor sensitivity by increased background noise when compared to acetonitrile and for next experiments the acetonitrile was selected. Collision energy (CE) applied in the collision cell (Q2) to the precursor ion to generate the product ion of the MRM transition was varied in the range 5-20 V. Collision energies between 5-15 V generated highest dissociation rate for all compounds (fig. 2a). CE generating highest S/N was selected as the optimal value. Similar procedure was applied to fragmentor voltage in the range 80-120 V. The maximum signal was obtained with fragmentor ranging in 80-110V (fig. 2b). Other ionization parameters were optimized by direct injection of analytical standards solutions. The parameters tested for ionization were: gas flow, gas temperature, capillary voltage, nebulizer pressure. The final chosen values were: 4000V, capillary voltage, 300°C drying gas temperature and 6 L/min drying gas flow. The same product ion was observed for all parabens. One product ion obtained was m/z 92, which corresponds to the loss of CO_2 group whereas the second product ion for parabens was m/z 136 which correspond to the loss of methyl, ethyl, propyl/isopropyl, benzyl group.

Ultrasonic extraction optimization

In order to reduce the interferences, the nature of the *extraction solvent and the volume required* for paraben extraction was tested by successively processing 0.5 g of lyophilized sludge to which 50µg/L standard internal was added. The sludge samples spiked with 25µg/L mixed of parabens (50ng/g) and non-spiked samples were analyzed. The concentrations detected in non-spiked sludge were subtracted from the results of spiked sludge. In this solvent selecting step, the cleanup of extract was not applied. According to the chemical properties of the target analytes, several extraction solvents were tested: methanol, acetone, acetonitrile and 50/50 (v/v) mixtures thereof. As

is well known, methanol is a very polar organic solvent with a strong ability to extract a wide range of chemical compounds from different types of samples. However, this solvent, in addition to the compounds of interest, co-extract other impurities such as pigments. These impurities can complicate the purification step because of matrix interference by reducing the precision of instrumental analysis. Because other solvents show lower extraction efficiencies (fig. 3), the mixture of methanol and acetone (1:1, v/v) was selected as the extraction solvent. This ensures simultaneously extraction of the studied parabens with good yields in the sludge samples by applying the described procedure. The solvent extraction volume was studied in the range 5-15mL (in steps of 5mL) and the extraction time was tested in the range 15-25 min (in steps of 5min). Extraction time higher than 15 min decreased the recovery efficiency. This could be explained by an increase of co-extracted interferences (humic acids), which generates a higher response of mass spectrometer. The solvent extraction volume higher than 5 mL decreased the recovery. The best extraction conditions were 5mL solvent extraction volume and 15min time extraction (table 3).

Method validation

The analysis method has been validated for sludge in terms of: linearity, limits of quantification, precision, accuracy. The calibration curves were plotted at 6 points in the operating range of 1 µg/L - 100µg/L. Each calibration level was analyzed in duplicate. In table 4 are presented correlation coefficients obtained for the calibration curves. Linearity correlation coefficient (R^2) has been used to evaluate the linearity domain. A very good linearity was observed for all compounds in the linear concentration range of R^2 ranging between 0.9959 and 0.9998. In order to ensure precise quantification, the variability of the method under the same conditions was assessed in a short time and over a longer time for a concentration of 50ng/g of analytes. 1 mL of 25 µg/L mixed standard solution and 1 mL of standard internal solution (EtPB-13C6) at 50 µg/L was added. The lyophilized sludge sample was first analyzed without standard addition and the detected concentrations were subtracted from spiked samples. The precision was determined from 3 samples of the same day (repeatability) and daily for 3 days (intermediate precision). Precision was expressed as a relative standard percentage (RSD%). RSD values obtained for repeatability ranged from 3.4 to 7.4% for all compounds. For intermediate precision experiments RSD was below 14.5%. Results obtained from repeatability and precision intermediate precision experiments indicate that the method is precise. Recovery of the method was studied by spiking known amounts of parabens (50ng/g) to three replicate sludge samples. Un-spiked samples were analyzed to detect potentials parabens and the positive results were subtracted. Table 4 presents the recovery results obtained for parabens that ranged from 71.1 to 109% with relative standard deviation (RSD) <14%. The obtained recoveries are similar with results reported in USA (78-113%) [7] and in Spain (80-125%) [10]. Also, similar LOQs were obtained for parabens determination in sludge in Spain (0.3-6 ng/g) [10]. Analyzing the results, we can see that possible losses due to sample pretreatment, extraction and variations in measurements due to matrix effects are satisfactorily corrected by the use of internal standards.

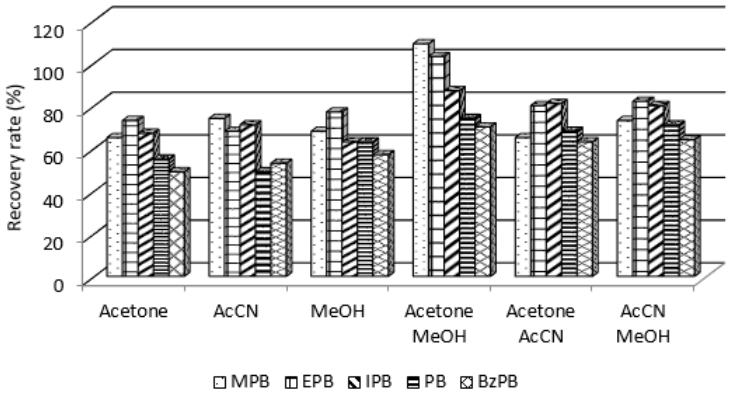

Fig. 3. Solvent selection for parabens extraction

Compound	Recovery rate (%)					
	Time of ultrasonic extraction			Volume of the extraction solvent		
	15 min	20 min	25 min	5ml	10ml	15ml
MPB	109	65.3	55.7	108.6	73.2	62.3
EPB	103.2	55.4	47.5	102.5	70.2	68.5
IPB	87.1	36.6	55.6	87.3	68.3	64.4
PPB	74.5	26.9	42.3	74.1	53.1	51.5
BPB	71.1	44.2	39.2	71.5	50.8	54.6

Table 3
RECOVERY RATE DEPENDING ON THE TIME OF ULTRASONIC EXTRACTION AND VOLUME OF THE EXTRACTION SOLVENT

Compound	R^2	Calibration range (µg/L)	Recovery rate± RSD (%)	Precision (%)		IQL (µg/L)	LOQ ng/g
				intra-day	inter-day		
MPB	0.9959	1-100	109 ± 14	3.7	14.5	0.5	1
EPB	0.9998	1-100	103.1 ± 9	3.4	9.4	0.6	1.2
IPB	0.9966	1-100	87.1± 9	6.3	8.8	0.5	1
PPB	0.9969	1-100	74.5 ± 13	7.4	13.1	1	2
BPB	0.9976	1-100	71.2 ± 10	6.5	9.7	0.2	0.4

Table 4
CORRELATION COEFFICIENTS (R^2), INTRA-DAY AND INTER-DAY PRECISION, RECOVERY, INSTRUMENTAL QUANTIFICATION LIMIT (LOQ)

Compound	Average	Minimum concentration	Maximum concentration	Detection frequency (%)
MPB	15.28	11.6	19.2	100
EPB	3.72	2.5	6.4	100
IPB	3.3	1.7	4.8	80
PPB	4.37	3.6	5.3	60
∑PB	24.26	19	32.7	-

Table 5
SUMMARY OF PARABENS CONCENTRATIONS DETECTED IN SEWAGE SLUDGE SAMPLES (NG/G D.W.)

Parabens occurrence in sludge samples

A total of 5 sludge samples from WWTP were analyzed for the detection of the five parabens. MPB and EPB were most frequently detected in each sludge sample (100% detection frequency, table 5) in concentration ranges of 11.6-19.2 ng/g d.w. and 2.5-6.4 ng/g d.w at averages of 15.28ng/g d.w and 3.72ng/g d.w. The second frequent paraben was IPB which was detected in 4 sludge samples with (frequency of 80%), at average of 3.3ng/g d.w. and in a range of 1.7-4.8 ng/g d.w. The third detected compound as frequency was PPB which was determined only in 3 investigated sludge samples (60% detection frequency) at concentrations ranging from 3.6 to 5.3ng/g.

The sum concentrations of all parabens for each sludge sample ranged between 19 ng/g dry weight and 32.7 ng/g dry weight. Benzyl-paraben, was not detected in any of the samples analyzed in this study. The physical-chemical properties such as solubility (160-5600mg/L) and Log K_{ow} (1.6-3.6) suggests that parabens present affinity to organic matter being adsorbed on sludge by hydrophobic interaction. This shows that parabens can be accumulated in sludge depending to chain length of alkyl derivate [1, 6]. The profile of sludge composition is similar with that one reported in some US WWTPs in which MPB was detected in the highest concentration range (24.3-68.8ng/g), EPB has the second concentration levels (1.6-12ng/g), PPB presented concentrations ranging from 0.36-4.64ng/g [16, 22].

Conclusions

In the present paper a chromatographic method was validated for the detection of five parabens in WWTP sludge. We proposed a pretreatment of solid samples which consist in sludge lyophilization followed by ultrasonic extraction with acetone/methanol (1/1). The next step was a clean-up using solid phase extraction with polymeric Strata X cartridges followed by LC-MS/MS determinations of parabens. The method was optimized by variation of their parameters which improved the recovery and the limit of quantitation. Good precision was obtained over the entire procedure. Finally, the method was successfully applied to real sludge samples collected from a municipal WWTP. Results showed the omnipresence of MPB and EPB at ng/g levels in all samples which were detected with average concentration of 15.3ng/g d.w. and 3.7ng/g d.w. The IPB and PPB were detected with average concentration of 3.3ng/g d.w. and 4.4 ng/g d.w.

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