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Animal Feeds Extracted from Excess Sludge by Enzyme, Acid and Base Hydrolysis Processes

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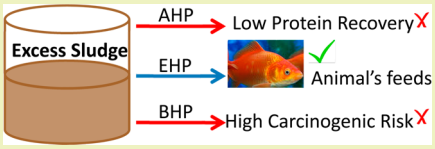
ABSTRACT: Eighteen polycyclic aromatic hydrocarbons (PAHs) and crude proteins were recovered by the disintegration of excess sludge (ES) through enzyme hydrolyzed process (EHP), acid hydrolyzed process (AHP) and base hydrolyzed process (BHP) followed by isoelectric precipitation, centrifugation and freeze-drying. The BHP was observed to have the highest total concentrations of PAHs in the crude proteins as compared to AHP and EHP, following: EHP (213.7 ng/g) < AHP (451.9 ng/g) < BHP (860.1 ng/g). The total carcinogenic PAHs ($\sum PAHs_{care}$) were found to be 34.6, 65.2, and 127.0 ng/g for EHP, AHP and BHP, respectively. The concentration of metals was found to be in the order of Fe > Zn > Mn > Cu > Pb > Ni > Cr > As > Hg > Cd in the ES samples before treatment. Except Zn, all the metals were in good agreement with the prescribed limits as defined by legal standard. Moreover, the concentration of all metal ions was found to be less or negligible in the EHP recovered proteins as compared to that in the BHP and AHP. The acute toxicity tests using Brocarded carp did not show any significant effects on the mortality, incidence of clinical signs, and body weight loss. During the 14 day experiment, the total concentrations of PAHs were in the range from 1.0 to 69.0 ng/g, whereas the carcinogenic PAHs in the carp fresh were found very low in the EHP recovered proteins. Based on these results, the EHP is an efficient and environmental friendly method to remove the PAHs as compared to BHP, for the production of animal feeds.



KEYWORDS: *Excess sludge, PAHs, Protein recover, Enzyme hydrolyzed, Gas chromatography−mass spectrometry*

INTRODUCTION

To date, a number of wastewater treatment plants (WWTPs) have been built up and successfully operated around the world. Due to rapid technical development and population growth, the volume of excess sludge (ES) produced by WWTPs has been increased in many folds. In addition, the stringent environmental regulations also limit their safe disposal.[1] The ES is considered as a major limiting factor for the successful operation of a WWTP, because the treatment, transportation, storage and final disposal of the ES account for approximately 50% of the operational costs.[2−4] Moreover, the sludge produced from WWTPs poses a major environmental problem due to its higher level of organic and heavy metals content,[5,6] pathogenic bacteria[7,8] and organic pollutants.[9−11] Consequently, to develop an economical and environmental friendly treatment method, for the sludge recovery and reusage as resources, is becoming more important.

The ES is a complex mixture of carbohydrates, amino acids, alcohols, volatile fatty acids, polymers and heteropolymers such as proteins, polysaccharides and lipids.[12−14] Proteins are the most abundant components of ES and account for approximately 60% among the three organic matters.[15] On the other hand, it is one of the most important nutritive substances in animal feeds, furnishing energy and nitrogen. The conversion of this waste biomass to higher-value products has been recognized as an attractive alternative waste management solution.[16,17]

As the early 1980s, the dry sludge has been reported to be applied to feed livestock without any other treatments, but this untreated sludge is not conducive to the growth of animals.[18] Because of the toxicity of heavy metals or other contaminants, animals feed by dry sludge showed weight loss.[19,20] Therefore, an efficient and inexpensive process to introduce no or less toxic content protein extraction is needed for ES to feed animals. In order to extract proteins, the destruction and dissolution of bacterial cell wall and intracellular materials in ES is the most important. Various chemical, physical and biological treatments such as alkali treatment,[21] ultrasonication[22,23] and enzyme treatment[24−26] have been reported to decompose the sludge folc structure to release the intracellular substances into the aqueous phase. For example, Dey et al.[24] found that the polysaccharides and sugar polymers were released significantly from extracellular polysaccharide (EPS) by the role of enzyme. Roman et al.[25] investigated the hydrolysis of enzyme such as cellulose enzyme and chain protease E in reducing the solid sludge particles and improving the sludge digestion. Yang et

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al.[26] proved the solubilization effect of the protease in combination with α-amylase for sludges. Chishti et al.[27] used ammonium sulfate (40%) and recovered 91% of proteins from ES. Most metals present in the primary sludge were removed from the recovered products, which comprised all the necessary amino acids, indicating a higher nutritional value to use as a feed for animals or as a nitrogen source for cotton and other cash crops.[28] Hwang et al.[29] used ultrasonication-assisted base hydrolysis to extract proteins from ES and reported the extraction rate as high as 80.5%. In addition, the coarse protein composition, toxic substances and acute toxicity on the rats were examined. The clinical symptoms, such as weight changes and mortality rate observed in the rats feed by the sludge proteins, showed that the proteins extracted from sludge could be used as animal feed safely. Therefore, it is possible to utilize the ES as the original material to generate raw proteins as animal feeds.

Polycyclic aromatic hydrocarbons (PAHs) are an important group of environmental contaminants. As many of these substances have a high toxicity, mutagenic and carcinogenic behaviors,[30] relatively stable and persistent nature in the environment, they are also regarded as persistent organic pollutants (POPs). PAHs have higher hydrophobicity and poor biodegradability, therefore their adsorption on the solid sludge particles is inevitable in WWTPs. Similarly, the heavy metals, 0.5−2% on wet basis and 4% on a dry sludge basis, were reported.[31] PAHs and heavy metals present in ES may accumulate in soil and be absorbed by the organism or migrate into groundwater, and in due course may harm the human being. Thus, the presence of PAHs and heavy metals is the primary problem to utilize sludge as fertilizer.

The present study aims at the identification and determination of 18 types of PAHs in the ES and the recovery of crude proteins by acidic, alkali and enzymatic hydrolysis process using a gas chromatography−mass spectrometry (GC−MS). The recovered proteins were obtained by disintegration of the ES through enzyme, acid and base hydrolyzed process followed by isoelectric precipitation, centrifugation and freeze-drying. The toxicants of the enzymatic proteins were analyzed, and the toxicity was assessed by Brocarded carp experiments. The concentration of metals in the ES and enzymatic hydrolysate was analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES).

MATERIALS AND METHODS

Chemicals. All the solvents such as dichloromethane, hexane, acetone and methanol were HPLC grade. Acetone, methanol and dichloromethane were supplied by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Hexane was obtained from Excellence J&K Scientific Ltd. (Beijing, China). Alkaline protease was purchased from Beijing Ao Bo Xing Universeen Bio-Tech Co., Ltd., which was available as an aqueous solution (activity of 25.1×10^4 U/g). Deionized water was produced by a Milli-Q system (Millipore Co., USA).

A mixture of polycyclic aromatic hydrocarbons named "610 PAH Solution" containing: benzo[e]pyrene, benzo[j]fluoranthene, benzo[k]fluoranthene, acenaphthene, acenaphthylene, anthracene, fluorine, naphthalene, phenanthrene, benzo[a]anthracene, benzo[a]pyrene, chrysene, fluoranthene, indeno[1,2,3-cd]pyrene, pyrene, benzo[b]fluoranthene, benzo[ghi]perylene, dibenz[a,h]anthracene, benzo[e]pyrene and benzo[j]fluoranthene) was obtained from o2si (Charleston, South Carolina, USA). The semivolatile internal standard mixture (2000 μg/mL in methylene chloride, purity higher than 98%) with components of acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, perylene-d_{12} and naphthalene-d_8 was also purchased from o2si. The internal standard solution used to spike all the samples prior to extraction was prepared at 4 μg/mL level in methylene chloride for each component. The calibration solutions were prepared by diluting the standard solution with dichloromethane and were stored at −20 °C.

Sampling Materials. The ES samples were obtained from the secondary sedimentation tank of a municipal WWTP (Shanghai, China) with a capacity of 1.5×10^4 m^3 per day. This plant used a typical anaerobic−anoxic−oxic process and the sludge was mechanically dewatered to 20−25% dry solids content by a belt press filter. The ES samples were maintained at 4 ± 0.5 °C in a fridge prior to use. The total nitrogen content was determined by the Kjeldahl method using 6.25 conversion coefficients and found to be 58.7% of protein (dried sludge, DS). A representative subsamples were taken for the present study. The samples were mixed, dried in a Desktop multifunction lyophilizer, ground and passed through a 74 μm sieve before use.

Protein Recovery Methods. Three sets of disintegration experiments such as enzyme, acid and alkali treatments were carried out. In brief, for the enzymatic treatment, the diluted suspension of ES was prepared by mixing the ES in distilled water (1:6) and then adding alkaline protease (2%, w/w) slowly to the ES suspension. After adjusting the pH at 8.0, the suspension was placed on a vibrating shaker at 55 °C for 8 h. At the end of the incubation, the solution was heated at 95 °C for 15 min and then centrifuged under a relative centrifugal force (RCF) of about 1450 g for 15 min. The supernatant was collected as the ES protein hydrolysate. For the acid treatment, the pH of diluted ES was adjusted to 0.5 using HCl (2.0 M) and the sludge was agitated by magnetic stirrer with condensate return flow under 121 °C for 5 h [28]. For the base treatment, the pH was adjusted to 13 with NaOH (1.0 M) and was stirred at 140 °C for 3 h [32]. After disintegration, the isoelectric precipitation was performed by adjusting the pH = 5.5 of the supernatant by using dilute HCl and NaOH solution. The crude proteins were recovered after shaking for 10 min and centrifuged at a RCF value of about 25,177 g for 30 min at 4 °C. Finally, the samples were labeled as enzymatic hydrolysis process (EHP) proteins, acidic hydrolysis process (AHP) proteins and alkaline hydrolysis process (BHP) proteins followed by lyophilize. The samples were grinded and stored in a desiccator at room temperature for further analysis.

Toxicity Test. The fishes were acclimatized in dechlorinated municipal water and fed with commercial fish food at 25 °C with light and dark ratio of 16:8 h for 2 weeks prior to test. For acute toxicity analysis of the EHP, the fishes were stopped feeding at least 1 day and subdivided into three groups according to their size, each group consisting of five fishes. The first group was set as control group, whereas the remaining groups were fed by 190 and 290 mg EHP recovered proteins, respectively. After 14 days of exposure, the fishes were sacrificed by execution. Finally, their muscular tissues were sampled from each pool followed by lyophilize, homogenized and maintained in a desiccator at room temperature for further chemical analysis.

Microwave Extraction. The microwave-assisted extraction was carried out using a MARS-6 laboratory microwave extraction system (CEM Inc. Matthew, North Carolina). The instrument is able to extract concurrently 40 samples under identical extraction conditions. Each freeze-dried sample (1 g) was weighed into an extraction vessel and supplied with 15 mL of extractant (1:1 dichloromethane/acetone). After the vessels were placed in the rotor, the samples were extracted under a power of 800 W using the following microwave program: 5 min ramp from room temperature to 120 °C and then 20 min held at 120 °C. After cooling, the vessel contents were filtered through 0.45 μm filter paper and the filtrate was concentrated to 1 mL at 30 °C using a gentle nitrogen stream.

Clean-up/SPME Extraction. The extraction process was followed by a cleanup step using a solid-phase microextraction (Visiprep DL, USA) with florisil (500 mg, 3 mL, GmbH, Germany) as absorbents. Florisil was pretreated with acetone (4 mL), vacuumed for about 5 min and then preconditioned by hexane (4 mL) under natural flow. The extracted sample (1 mL) was transferred to the column and the

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extraction vessel was transferred to the column followed by washing with hexane−acetone (3 mL). The column was placed in a multiport vacuum manifold, and hexane (15 mL) was applied to remove the aliphatic hydrocarbons. Then, the eluents containing PAHs were collected by eluting with 70 mL of dichloromethane/hexane (3:7, v:v) and were concentrated to 0.5 mL under a gentle purified N_2 stream. 5 μL internal standards (100 μg/mL) were added to the sample prior to the GC/MS analysis.

Analytical Instrument and Apparatus. The PAHs were analyzed using a GC/MS (Shimadzu, QP2010 Ultra) with a 30 m × 0.32 mm × 0.25 μm film thickness HP-5 MS column (J&W Scientific, USA). The GC/MS conditions for the sample analysis were as follows: the injection port, interface line and ion source temperature were maintained at 300, 280 and 20 °C, respectively. Splitless injection of 1 μL sample was carried out. Helium was used as carrier gas at 1.2 mL/min. The GC oven temperature was maintained at 70 °C for 2 min, increased to 260 °C at a rate of 10 °C/min and maintained for 8 min, then to 300 °C at 5 °C/min and maintained for 5 min. The whole process time (42 min) and a solvent delay time (4 min) were selected. The mass spectrometer was operated in an electron impact ionization mode (70 eV). The scan ions were ranged from 28 to 500 m/z, and the dwell time per ion was 0.3 s. The substance analysis was undertaken with reference to the NIST11 mass spectral library database. The quantitation and quantification criteria included the confirmation of retention times and isotope ratios of the labeled standards and respective analyte. The GC retention time, important ions (m/z) and internal standard for the constituents of PAHs are listed in Table 1. A SIM program was used to screen four groups of pollutants having specific ions for each compound and a specific group start time (Table 2).

Identification and Quantification. The PAHs were confirmed by their retention times, the identification of target and qualifier ions and the determination of qualifier to target ratios. The retention time must be within ±0.3 min of the expected time and the qualifier-to-target ratios are within a 20% range for positive confirmation.

Table 1. Identification of 18 PAHs and 5 Deuterium (d) Labelled Internal Standard

S. no	PAHs	t_R	m/z[a]	internal standard
1	naphthalene-d_8	8.205	136,135	
2	naphthalene	8.255	128,129,102	naphthalene-d_8
3	acenaphthylene	12.235	152,151,153	acenaphthene-d_{10}
4	acenaphthene-d_{10}	12.605	164,162	
5	acenaphthene	12.690	154,153,152	acenaphthene-d_{10}
6	fluorene	14.015	166,165,167	acenaphthene-d_{10}
7	phenanthrene-d_{10}	16.390	188,184	
8	anthracene	16.460	178, 152,176	phenanthrene-d_{10}
9	phenanthrene	16.585	178,152,176	phenanthrene-d_{10}
10	fluoranthene	19.500	202,203,101	chrysene-d_{12}
11	pyrene	20.070	202,203,101	chrysene-d_{12}
12	benz(a)anthracene	23.610	228,229,226	chrysene-d_{12}
13	chrysene-d_{12}	23.640	240,125	
14	chrysene	23.740	228,229,226	chrysene-d_{12}
15	benzo(b)fluoranthene	28.980	252,253,126	perylene-d_{12}
16	benzo(k)fluoranthene	29.005	252,253,126	perylene-d_{12}
17	benzo(j)fluoranthene	29.040	252,253,126	perylene-d_{12}
18	benzo(a)pyrene	30.630	252,253,126	perylene-d_{12}
19	benzo(e)pyrene	30.935	252,253,126	perylene-d_{12}
20	perylene-d_{12}	31.255	264	
21	indeno(1,2,3-cd) pyrene	36.730	276,277,138	perylene-d_{12}
22	dibenz(a,h)anthracene	36.920	278,276,138	perylene-d_{12}
23	benzo(ghi)perylene	37.860	276,277,138	perylene-d_{12}

[a]Target ions are underlined.

A calibration solution was performed with the PAH standards and the deuterated PAHs solution as internal standard. The linear range was established by a seven point calibration curve in the range of 0.01−1.0 μg/mL and each calibration level was spiked with deuterated PAHs (0.10 μg/mL) as an internal standard. The limit of detection (LOD) was calculated according to the 3 S_b/m criterion, and the values of LOQ are defined as ten times the signal-to-noise ratio,[33,34] where m is the slope of the calibration curve and S_b is the standard deviation.

Metals Analysis. To measure the concentrations of heavy metals, the ES and the recovered freeze-dried pellet samples were transferred to a mortar and grinded to fine powders. To determine the overall content of heavy metals, the samples were placed into 100 mL beaker. Next, 18 mL of HNO_3 was added and covered with watch-glass for overnight. The sample was digested nearly dry on an electric hot plate at a low temperature, heated up after adding 5 mL of $HClO_4$ until the solution evaporated and dried. The samples were transferred into 100 mL bottles with deionized water and stored at 4 °C for further analyses of the heavy metals: zinc, copper, nickel, cadmium, lead and chromium with an inductively coupled plasma atomic emission spectrometry (ICP-AES) (Prodigy, Leeman, USA). Three different samples of the same sludge were prepared for analyses.

RESULTS AND DISCUSSION

ES Characteristic. The ES was characterized by a weak acid (pH 6.85) and water (83.55%). The dry sludge consists of 73.8% organic matter, which contains protein (58.1%), carbohydrate (10.2%) and lipids (0.6%). The protein and carbohydrate were reported to be the main organic constituents of the domestic sludge [35]. Liu et al.[28] reported that the waste sludge consisted of 41.8% protein and 18.5% carbon hydrate in dry sludge.

Identification of PAHs. The total ion chromatogram (TIC) from the GC/MS analysis of the PAHs standard solution is presented in Figure 1. The established chromatographic method is shown to be a good way to separate the target components, providing the quantitative analysis of each component of the PAHs.

CHARACTERIZATION AND VALIDATION OF THE METHOD

Linearity, Limits of Detection (LOD) and Quantification (LOQ). A multipoint calibration with seven standard solutions of different concentration levels, in the range of 10−1000 ng/mL, appropriate to the levels found in our samples, was used. The deuterated internal standard at a concentration of 100 ng/mL was added to the standard solutions prior to injection. A good linearity of the calibration curves was obtained in the range of 10−1000 ng/mL. The correlation coefficient was 0.98 or higher for most of the PAHs analyzed. In Table 3, the correlation coefficients and regression equations of the calibration curves are listed together with the LOD and LOQ values corresponding to PAHs with the lowest concentration. Low LODs were obtained due to the high sensitivity of the GC−MS-SIM, allowing the quantification of PAHs at the levels present in the ES samples. The LODs and LOQs are in the range from 1.82 to 75.15 pg/mL and from 6.06 to 250.50 pg/mL, respectively. The range of LODs achieved is in the lower end of those obtained by other researchers.[36−38]

Recoveries of PAHs. The recoveries of the PAHs were calculated using their response factor related to their own internal standards. All the samples were analyzed in triplicate. The experimental mean concentration obtained for each PAH is presented in Table 4. The recovery provides an estimate of

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Table 2. SIM Program for Monitoring Various Toxicants in the Samples[a]

group	time (min)	m/z	PAHs
1	4	128,136(I.S.$_1$),152,154, 166,164(I.S.$_2$), 178,188(I.S.$_3$)	naphthalene-d_8, naphthalene, acenaphthylene, fluorene, acenaphthene-d_{10}, acenaphthene,phenanthrene-d_{10}, anthracene, phenanthrene
2	18	202,228,240(I.S.$_4$)	fluoranthene, pyrene, chrysene-d_{12}, chrysene, benz(a)anthracene
3	26	252,264(I.S.$_5$),276,278	benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(j)fluoranthene, benzo(a)pyrene, benzo(e)pyrene, indeno(1,2.3-cd)pyrene, perylene-d_{12}, dibenz(a,h)anthracene, benzo(ghi)perylene

[a]I.S.$_1$ = naphthalene-d_8; I.S.$_2$ = acenaphthene-d_{10}; I.S.$_3$ = phenanthrene-d_{10}; I.S.$_4$ = chrysene-d_{12}; I.S.$_5$ = perylene-d_{12}.

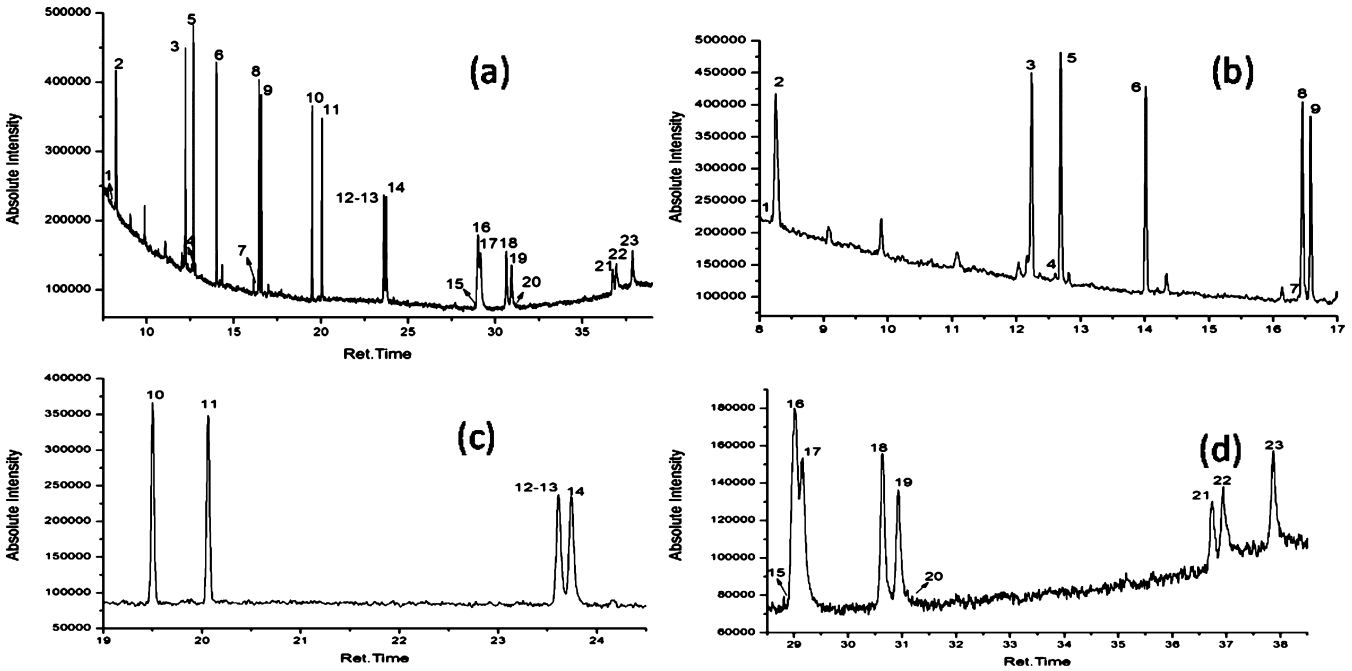

Figure 1. GC/MS spectrum of (a) PAHs standard; (b) nine PAHs (1, naphthalene-d_8; 2, naphthalene; 3, acenaphthylene; 4, acenaphthene-d_{10}; 5, acenaphthene; 6, fluorene; 7, phenanthrene-d_{10}; 8, anthracene; 9, phenanthrene); (c) PAHs (10, fluoranthene; 11, pyrene; 12, benz(a)anthracene; 13, chrysene-d_{12}; 14, chrysene) and (d) the PAHs (15, benzo(b)fluoranthene; 16, benzo(k)fluoranthenel 17, benzo(j)fluoranthene; 18, benzo(a)pyrene; 19, benzo(e)pyrene; 20, perylene-d_{12}; 21, indeno(1,2.3-cd)pyrene; 22, dibenz(a,h)anthracene; 23, benzo(ghi)perylene).

Table 3. Calibration Curve and Limits of Detection and Quantification (LOD, LOQ pg/mL) for 18 PAHs

PAHs	calibration data (equation)	r^2	LOD (pg/mL)	LOQ (pg/mL)
naphthalene	$y = 2961.6x - 11499$	0.9991	2.89	9.62
acenaphthylene	$y = 2001.9x - 19430$	0.9986	2.99	9.97
acenaphthene	$y = 1488.7x - 12506$	0.9986	1.82	6.06
fluorene	$y = 1324.5x - 11696$	0.9978	3.15	10.50
anthracene	$y = 1175.2x - 15626$	0.9935	5.03	16.77
phenanthrene	$y = 1466.8x - 36110$	0.9914	2.67	8.91
fluoranthene	$y = 1057.6x - 17886$	0.9922	3.65	12.17
pyrene	$y = 1065.8x - 18545$	0.9920	4.62	15.41
benz(a)anthracene	$y = 342.34x - 8569.2$	0.9879	8.66	28.88
chrysene	$y = 695.34x - 15732$	0.9912	7.75	25.84
benzo(b&k)fluoranthene	$y = 706.46x - 12890$	0.9856	4.87	16.22
benzo(j)fluoranthene	$y = 812.19x - 30037$	0.9792	4.63	15.43
benzo(a)pyrene	$y = 518.77x - 12228$	0.9876	8.90	29.66
benzo(e)pyrene	$y = 460.21x - 11470$	0.9805	6.80	22.66
indeno(1,2.3-cd)pyrene	$y = 258.59x - 4640.7$	0.9952	75.15	250.50
dibenz(a,h)anthracene	$y = 446.63x - 22787$	0.9700	2.06	6.86
benzo(ghi)perylene	$y = 560.72x - 18300$	0.9800	3.61	12.02

the accuracy of the whole method, and the relative standard deviation (RSD) shows the precision of the determination of each PAH. The extraction used in this study provided 77.5−107.4% recovery of each pollutant from the dichloromethane samples in the presence of the internal standards with RSD lower than 4.86%. Thus, almost all the pollutants present in the dichloromethane were extracted by the SPE process. The recoveries of PAHs were between 75.3% for naphthalene and

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Table 4. Percentage Recovery of Each Compound from Dichloromethane, ES and Brocarded Carp Samples

PAHs	dichloromethane (n = 3)		ES (n = 3)		brocarded carp (n = 3)	
	recovery (%)	RSD[a]	recovery (%)	RSD	recovery (%)	RSD
naphthalene	77.45	4.86	75.33	4.37	72.18	5.36
acenaphthylene	99.02	2.03	102.51	1.51	95.23	1.63
acenaphthene	91.77	1.86	99.87	2.06	105.04	2.58
fluorene	96.36	1.78	93.69	1.99	96.91	1.92
anthracene	89.69	2.35	105.18	2.17	88.79	3.55
phenanthrene	101.02	2.14	101.99	2.68	97.82	2.74
fluoranthene	97.15	2. 23	99.72	3.32	92.07	3.03
pyrene	98.07	2.09	107.14	2.69	85.56	2.96
benz(a)anthracene	101.68	1.87	89.13	1.93	97.28	1.89
chrysene	88.79	1.97	92.86	1.74	88.75	2.15
benzo(b&k)fluoranthene	95.33	4.28	109.35	2.85	105.27	3.09
benzo(j)fluoranthene	94.72	3.14	96.08	3.59	89.33	1.52
benzo(a)pyrene	91.85	2.71	94.51	2.78	100.75	2.64
benzo(e)pyrene	95.18	3.05	102.45	4.56	87.59	3.93
indeno(1,2.3-cd)pyrene	99.53	3.69	91.18	3.29	107.69	4.87
dibenz(a,h)anthracene	100.82	2.53	87.29	3.55	98.15	3.49
benzo(ghi)perylene	107.37	4.37	90.93	4.08	95.72	3.58

[a]Relative standard deviation (%).

109.4% for benzo(b&k)fluoranthene with a RSD lower than 4.6%. The extraction of the spiked fancy carp samples provided 72.2−107.7% recoveries with a low RSD, ranging from 1.5 to 5.4%. The observed lowest recovery of naphthalene, consistent with the previous studies,[39−41] is due to the fact that naphthalene is PAHs, which contained only two fused benzene rings and its high water solubility (30 mg/L) and volatile.

Levels of PAHs. The concentrations of the identified PAHs in the ES before and after treatment are summarized in Table 5. In all the samples of ES, the concentrations of the five PAHs such as anthracene, fluorene, phenanthrene, acenaphthene and naphthalene were detected in higher concentration with a mean concentration of 291.9, 238.6, 234.7, 216.8 and 208.3 ng/g, respectively. Fluoranthene, pyrene, benz(a)anthracene, chrysene and benzo(b&k)fluoranthene were detected at low levels, with mean concentrations ranging from 26.5 to 47.5 ng/g. Whereas benzo(b&k)fluoranthene was calculated by grouping together benzo(b)fluoranthene and benzo(k)fluoranthene. It was also found that low concentrations of six ring PAHs, such as indeno[1,2,3-cd]pyrene and benzo[ghi]perylene have a mean value of 55.1 and 39.9 ng/g, respectively. PAHs, like acenaphthylene and benzo(a)pyrene and dibenz(a,h)-anthracene were found to be in the least concentrations. Whereas others such as benzo(j)fluoranthene and benzo(e)-pyrene were not detected in all the sludge samples. Low molecular weight PAHs (2−3 rings) were abundant in ES with highest concentrations in anthracene, fluorene, phenanthrene, acenaphthene and naphthalene. According to previous studies,[42,43] 2−3 rings PAHs are mainly originated from low- or moderate-temperature combustion process (such as coal burning). Phenanthrene typically comes from the petroleum products, and the road traffic is a significant source of PAHs originated from vehicular exhaust.[44,45] The compositional pattern of PAHs by ring size in the urban WWTP confirmed that the PAHs were manipulated by both coal combustion process and road traffic.

In the present studies, the total PAHs concentration (sum of the 18 congeners, ∑PAHs) in the ES samples was 1545.85 ng/g (1.546 mg/kg), among them, the sum of total carcinogenic PAHs ($\sum PAHs_{care}$) was found to be 241.3 ng/g. The reported range of the total PAHs concentrations in the ES was 1−50 mg/kg and in most of the studies was found in the range of 1−10 mg/kg. However, higher concentration (121 mg/kg) of the total PAHs was also reported.[46−51] The total PAHs found in other Chinese cities, such as Beijing, Guangzhou and Zhuhai are between 30 to 70 mg/kg, except Shenzhen with a reported lowest 1.39 mg/kg.[52] The total PAHs concentration found in those work in Shanghai is very similar to the PAHs value reported by other researchers.[53] In comparison with the PAHs values reported in the literature, the concentration found in Shanghai was clearly lower than those found in Beijing, Guangzhou and Zhuhai.

Table 5. Concentrations (ng/g) of 18 PAHs in ES and Protein

PAHs	ES	EHP	BHP	AHP
naphthalene	208.34	18.07	186.53	49.87
acenaphthylene	20.89	10.82	11.78	7.04
acenaphthene	216.75	21.41	129.08	87.134
fluorene	238.57	34.41	117.88	67.43
anthracene	291.90	31.42	145.28	82.44
phenanthrene	234.68	43.64	107.30	56.32
fluoranthene	47.53	8.27	18.63	17.10
pyrene	45.88	11.11	16.59	19.37
benz(a)anthracene*	45.34	14.10	28.45	14.39
chrysene*	38.15	6.86	24.70	14.90
benzo(b&k)fluoranthene*	26.49	1.48	17.81	9.58
benzo(j)fluoranthene*	Nd[a]	Nd	14.78	5.12
benzo(a)pyrene*	21.82	Nd	Nd	Nd
benzo(e)pyrene*	Nd	Nd	Nd	Nd
indeno(1,2.3-cd)pyrene*	55.10	5.35	17.56	10.55
dibenz(a,h)anthracene*	14.50	1.97	10.30	1.99
benzo(ghi)perylene*	39.91	4.80	13.43	8.64
∑PAHs	1545.85	213.71	860.10	451.87
$\sum PAHs_{care}$[b]	241.31	34.56	127.03	65.17

[a]Not found. [b]$\sum PAHs_{care}$ is the total of carcinogenic PAHs, which are marked by an asterisk.

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The alkaline thermal hydrolysis is considered as an efficient sludge treatment[54,55] followed by acid hydrolysis, and enzymatic hydrolysis. Nevertheless, the present study revealed that the BHP has a higher concentration of PAHs in crude proteins as compared to AHP and EHP (Table 5), the total PAHs concentration (∑PAHs) in all three treatments was found to be in the following order: EHP (213.7) < AHP (451.9) < BHP (860.1). Whereas the total carcinogenic PAHs ($\sum PAHs_{care}$) was found to be 34.6, 65.2 and 127.0 ng/g for EHP, AHP and BHP, respectively. On the basis of these results, it can be suggested that the EHP is an efficient and environmental friendly method to remove the PAHs as compared to BHP, for the production of animal feeds.

In the present study, the crude protein extracted from ES by enzymatic method was tested as Brocarded carp feed. No death or clinic symptoms were found in the experiment period (14 days). After the experiment, the content of PAHs in Brocarded carp body was investigated, Table 6. The results showed that

Table 6. Concentrations of (ng/g) 18 PAHs in the Brocarded Carp Body

PAHs	carp unfed	control group	Group I	Group II
naphthalene	62.45	58.69	55.57	67.81
acenaphthylene	18.98	19.79	Nd[a]	19.50
acenaphthene	63.93	63. 96	79.98	83.39
fluorene	59.51	60.86	67.98	68.95
anthracene	43.18	45.00	53.54	52.36
phenanthrene	30.80	29.71	27.68	27. 90
fluoranthene	11.74	10.54	11.26	12.62
pyrene	8.88	8.65	13.11	10.14
benz(a)anthracene*	15.64	12.42	10.80	18.86
chrysene*	9.13	6.60	7.42	10.86
benzo(b&k)fluoranthene*	Nd	8.38	5.00	10.03
benzo(j)fluoranthene*	Nd	Nd	Nd	Nd
benzo(a)pyrene*	3.27	4.98	Nd	12.75
benzo(e)pyrene*	10.66	15.81	Nd	15.61
indeno(1,2.3-cd)pyrene*	8.86	3.86	Nd	7.50
dibenz(a,h)anthracene *	4.44	1.03	3.95	6.22
benzo(ghi)perylene*	4.61	3.77	Nd	8.86
∑PAHs	356.10	354.06	336.30	433.35
$\sum PAHs_{care}$[b]	56.61	56.85	27.17	90.69

[a]Not found. [b]$\sum PAHs_{care}$ is the total of carcinogenic PAHs, which are marked by an asterisk.

the concentrations of 18 PAHs in the carp body were in the range from 1.0 to 69.0 ng/g. The concentrations of naphthalene, acenaphthene, anthracene and fluorine were found to be relatively higher than other PAHs, this may be due to the low number rings of PAHs, which have higher water solubility, volatility and bioaccumulation. The concentrations of carcinogenic PAHs in the carp flesh were generally low. There was an unobvious trend that the concentrations of PAHs in the carp body increased at a high level of feed. Thus, the EHP extracted from ES is observed to have no effects on the mortality, incidence of clinical signs, body weight changes and necropsy findings. The concentrations of PAHs in the carp body were also significantly lower than the national food hygiene standards after they were feed up with EHP that came from the ES.

Mechanism of Enzymatic Hydrolysis of ES. The kinetics of ES enzymatic hydrolysis by alkaline protease was evaluated at 55 °C and pH 8. Various concentrations (4.0−50.0 g/L) of ES solution were used as substrate. Michaelis−Menten model was considered for the kinetic analysis and Lineweaver−Burke plot was used to assess the values of Michaelis constant (K_m) and maximum effective velocity (V_{max}). The resulting correlation coefficient was 0.998 and the kinetic analysis showed that K_m and V_{max} values were 34.9 g/L and 32.2 g/(L·min). The catalytic kinetic of the alkaline protease under various casein concentrations (2.0−20.0 g/L) as substrate has been reported.[56] The kinetic analysis showed that the apparent K_m for the alkaline protease under ES was about 7.2 times higher than that found when casein as substrate. This increase in apparent K_m value might be either due to the lower accessibility of the substrate or to the protease and/or inhibition effect from ES component, such as heavy metals, etc.

Metal Analysis. The contents of the identified metals in the ES, EHP, BHP and AHP are presented in Table 7. The

Table 7. Concentrations of Heavy Metals

	unit (mg/kg)				
element	ES	EHP	BHP	AHP	legal standard
Cu	260.63	1.059	218.39	189.14	[b]
Fe	10247.77	13.45	5021.42	4283.95	
Zn	913.47	0.2887	483.86	405.49	
Mn	488.60	1.0021	301.93	268.28	
Ni	47.59	0.0743	29.38	21.67	
Pb	53.38	1.2845	32.74	19.53	≤5
Cd	0.0058	≤0.001	≤0.001	≤0.001	≤0.5
Cr	0.5050	≤0.001	≤0.001	≤0.001	≤10
Hg	0.0289	≤0.001	≤0.001	≤0.001	≤0.1
As	0.0295	≤0.001	≤0.001	≤0.001	≤2

[a]The lowest in the hygienic standard for feeds in the people's Republic of China (GB 13078-2001). [b]Not prescribed in the regulation.

concentrations of metals were found to be in the order of Fe > Zn > Mn > Cu > Pb > Ni > Cr > As > Hg > Cd in the ES samples before treatment. Except Zn, all the metals were in good agreement with the prescribed limits of "Control standards for pollutants in sludge for agricultural use" (GB4284-84) enacted in 1984, China. Moreover, it is noted that the concentrations of all metal ions were found to be less or negligible in the EHP recovered proteins as compared to the BHP and AHP (Table.7). In addition, the concentrations of the toxic heavy metals (lead, chromium, cadmium, mercury and arsenic) were below the prescribed limit of the hygienical standard for the feeds in China (GB 13078-2001). Hence, it is suggested that the EHP crude protein process is technically feasible to be used as animal food.

CONCLUSIONS

In this study, a rapid and simple process for the determination of 18 PAHs in the ES and recovered crude proteins by acidic, alkali and enzymatic hydrolysis process using a GC−MS was discussed. The recovered proteins from BHP have the highest total concentrations of PAHs in crude proteins as compared to those from AHP and EHP, following the order: EHP (213.7) < AHP (451.9) < BHP (860.1). Whereas, the total carcinogenic PAHs ($\sum PAHs_{care}$) were found to be as 34.6, 65.2 and 127.0 ng/g for the proteins recovered by EHP, AHP and BHP, respectively. The concentrations of heavy metals in the ES samples were measured and found that all the metals were in good agreement with the prescribed limits of legal standard,

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apart from Zn. And compared with proteins by BHP and AHP, the concentrations of metal ions in the recovered proteins by EHP were found to be less or negligible. In most of the examined sludge samples, anthracene was the predominant compound, followed by fluorene, phenanthrene, acenaphthene and naphthalene. The acute toxicity tests using Brocarded carp did not show any significant effects on the mortality, incidence of clinical signs and body weight loss. During the experiment period of 14 days, the total concentrations of PAHs were in the range from 1.0 to 69.0 ng/g, whereas the carcinogenic PAHs in the carp fresh were also found very low in the EHP recovered proteins. On the basis of these results, the EHP is an efficient and environmental friendly method to remove the PAHs as compared to the BHP, for the production of animal feeds.

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Notes

The authors declare no competing financial interest.

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