Determination of 16 Polycyclic aromatic hydrocarbons in shellfish by HPLC-VWD/FLD

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Abstract: A method has been developed for the analysis of 16 polycyclic aromatic hydrocarbons(PAHs) in shellfish sample. The sample was ultrasonically extracted, separated through Florisil and MIP-pahs column, enriched with vacuum steaming-nitrogen blowing, and determined by HPLC- VWD/FLD. The pre-treatment method was optimized. At the spiked level of 0.5, 1.5 and 2μg/kg, the average recoveries of 16 PAHs were 58.1%-89.6%, 60.6%-88.1% and 72.3%-98.0%; and the relative standard deviations(rsds) were 0.23%-11%, 1.3%-13%, and 1.5%-14%, respectively, in Mytilus coruscus. The detection limits of the method for 16 PAHs were 0.5μg/kg. This method was used for the analysis of 16 PAHs in Ostrea gigas Thunberg, Meretrix meretrix and Sinonovacula constricta.

Keywords: Polycyclic aromatic hydrocarbons(pahs); HPLC; Florisil; MIP-pahs; Shellfish

Chinese Library Classification Number: O657.7; O657.3 Document identification code: A

Article No.: 1000-0720(2019)07-0828-06

Received on: 2018-08-26

Fund Project: supported by the risk assessment project of the Ministry of agriculture (gjfp201700903)

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Polycyclic aromatic hydrocarbons (pahs) are a class of compounds composed of two to seven fused aromatic rings with persistence. Long distance mobility. Lipophilicity and "three toxicities" widely exist in environmental media [1-3].

PAHs not only bring long-term harm to aquatic ecosystems, but also threaten human health through the food chain [4].

The sea is the key area of PAHs pollution, and shellfish are the main organisms with PAHs enrichment in marine organisms. The filtration feeding characteristics of shellfish and the low bioavailability of PAHs in their metabolic system lead to the easy enrichment of more PAHs in their bodies. It is of great significance to study the pollution of PAHs in shellfish and its health risk assessment.

At present, the main detection methods of PAHs in aquatic products are gas chromatography mass spectrometry (GC-MS)[5, 6] and high performance liquid chromatography (HPLC)[7-9]. GC-MS is a selective ion scanning method for trace analysis of pahs, but the pretreatment is cumbersome and the chromatographic column temperature is limited, so it is difficult to separate PAHs with high boiling point and similar structure. HPLC is relatively simple, sensitive, selective and accurate, and is widely used in the detection of PAHs in aquatic products. Because shellfish contain protein. For impurities such as lipids, Florisil should be used. C18 and other purification treatments remove impurities, thereby reducing the matrix effect [10]. In addition, under nitrogen blowing or vacuum concentration conditions, fluorene. PAHs with low boiling points such as phenanthrene are easy to be transferred to the air, thus reducing their recovery [5].

This study aims to establish an effective method for the analysis of PAHs in shellfish by high performance liquid chromatography Ultraviolet/fluorescence (hplc-vwd/fld) combined with external standard method. From chromatographic conditions. The purification column type and concentration conditions are optimized in order to speed up the process. Accurately analyze 16 kinds of PAHs in shellfish and other aquatic products to provide reference.

1 Experimental part

1.1 Instruments and reagents

Agilent 1100 high performance liquid chromatograph (Agilent); agilent Zorbax eclipse PAH column (4.6 mm × 150 mm, 3.5μm)(Agilent); CF16 RXII high-speed freezing centrifuge (Hitachi Group of Japan); MIP-PAHs special column (Shanghai Anpu experimental technology company); N-EVAP nitrogen blowing concentrator (American organization company); vacuum rotary vaporizer (Swiss Buqi laboratory equipment Trading Co., Ltd.).

Acetonitrile. Dichloromethane. Methanol (chromatographically pure, Baker); 16 kinds of PAHs single standard and mixed standard solutions: naphthalene (NA). Acenaphthene (AC). Acenaphthene (ACL). Fluorene (FL). Phenanthrene (PHE). Anthracene (AN). Fluoranthene (FA). Pyrene (py). Benzo [a] anthracene (BAA). Chr. Benzo [b] fluoranthene (bbfa). Benzo [k] fluoranthene (bkfa). Benzo [a] pyrene (BAP). Dibenzo [a, h] anthracene (dbaha). Benzo [g, h, i] perylene (bghip). Indene benzene [1, 2, 3-cd] pyrene (IP), concentration 10μg/mL (American chromatography Corporation).

1.2 Pretreatment of shellfish samples

1.2.1 Extraction of polycyclic aromatic hydrocarbons from shellfish

Shellfish samples are shelled and edible parts are taken. Use a meat grinder to grind it into a pulp, put it into a clean plastic bag, and store it at -20℃.

Weigh 5.00 g (accurate to 0.01 g) homogenate sample, put it into a 50mLcentrifuge tube, add 5mL ultra pure water and 15mL acetonitrile respectively, vortex mix for 1 min, and ultrasonic for 10 min. Then, 6 g of anhydrous $MgSO_4$ and 1.5 g of anhydrous sodium acetate were added and vortex mixed for 1 min. Finally, at 4℃, 8000 r/min, centrifugation for 10 min, collect the acetonitrile layer for purification.

1.2.2 Purification and concentration

The PAHs extract was loaded onto the Florisil column activated by acetonitrile, washed with 6mLof acetonitrile, collected in a 100mLchicken heart bottle, and concentrated to about 10mL(130 mbar vacuum, 35℃ water bath) by vacuum spinning.

Take 6mLof methylene chloride and 6mLof methanol respectively to activate the equilibrium MIP-PAH special column. Slowly load the above concentrated solution onto the MIP PAH column, and then rinse the small column with 6mLof methanol to discard the effluent. Finally, 10mLof methylene chloride was eluted and collected in a 100mLchicken heart bottle. Use vacuum rotary evaporation (280 mbar, 35℃ water bath) to concentrate to about 5 mL, and then adjust the vacuum degree to about 130 mbar to concentrate to about 2 mL. Transfer the concentrated solution to a 5mLglass graduated centrifuge tube, and then clean the spinning flask with 2mLacetonitrile, and combine the two. Blow nitrogen slowly at 35℃ to about 0.8 mL, dilute to 1mLwith acetonitrile, and pass 0.22μm organic microporous filter membrane, to be tested. At the same time, make reagent blank.

1.3 Drawing of standard curve

1.3.1 Configuration of standard stock solution

Take 0.5mLof 16 kinds of PAHs single standard solution and mixed standard solution (10μg/mL), respectively, add to a 25mL Brown volumetric flask and dilute with acetonitrile to prepare a single standard and mixed standard stock solution with a concentration of 200 ng/mL, and store at -18℃.

1.3.2 Drawing of standard curve of external standard method

Transfer 0.05, 0.25, 0.5, 1, 2.5mLof PAHs mixed standard stock solution (200 ng/mL) into a 10mLBrown volumetric flask, fix the volume with acetonitrile, prepare a series of standard working solutions with a concentration of 1, 5, 10, 20, 50 ng/mL, and analyze on the machine. The retention time was used for qualitative analysis, and the standard curves were drawn with the mass concentration of 16 PAHs as the abscissa and the peak area as the ordinate.

1.4 Chromatographic analysis conditions

Chromatographic column: Zorbax eclipse PAH column (4.6 mm × 150 mm, 3.5μm); column temperature 25℃; injection volume 10μL; flow rate: 1.2 mL/min; mobile phase: A is ultra pure water, B is acetonitrile, and the gradient elution procedure is detailed in Table 1. The detection wavelength of UV detector is 229 nm; the detection wavelength of the fluorescent detector adopts the program timing control mode, and the corresponding excitation wavelength (Ex) and emission wavelength (EM) are shown in Table 2.

2 Results and discussion

2.1 Optimization of chromatographic separation and detection conditions

In this experiment, 20 ng/mL single standard and mixed standard solutions were used as samples, and Agilent Zorbax eclipse PAH chromatographic column (4.6 mm) was used × 150 mm, 3.5μm)And the corresponding gradient elution procedure. Considering that the maximum pressure on the chromatographic column is ≤ 400 MPa and the polarity of PAHs is small, three elution procedures are compared in this experiment, and the elution procedures are shown in Table 1. The retention time of 16 PAHs components on the chromatogram was determined by single standard injection.

Table 1 Procedure of gradient elution for pahs

Procedure 1			Procedure 2			Procedure 3		
RT/min	A/%	B/%	RT/min	A/%	B/%	RT/min	A/%	B/%
0	50	50	0	50	50	0	50	50
2	50	50	2	50	50	2	50	50
18	100	0	22	100	0	22	100	0
26	100	0	28	100	0	29	100	0
27	50	50	30	50	50	30	50	50
35	50	50	38	50	50	38	50	50

The experiment shows that in program 1, when the proportion of acetonitrile reaches 100%, the pump pressure of liquid chromatography will gradually increase with the increase of injection times, and the corresponding retention time of several PAHs will drift, resulting in their inability to peak within the corresponding wavelength range; in procedure 2, when acetonitrile reaches 100% in 22 minutes and remains for 6 minutes, a heteropeak (on the right) will appear at the peak of IP, interfering with its qualitative and quantitative. After adjustment, the maintenance time of 100% acetonitrile is extended to 7 min (see Table 1, procedure 3), which can significantly separate PAHs in the mixed standard solution and stabilize the peak. See Figure 1 for the details of the determination chromatogram.

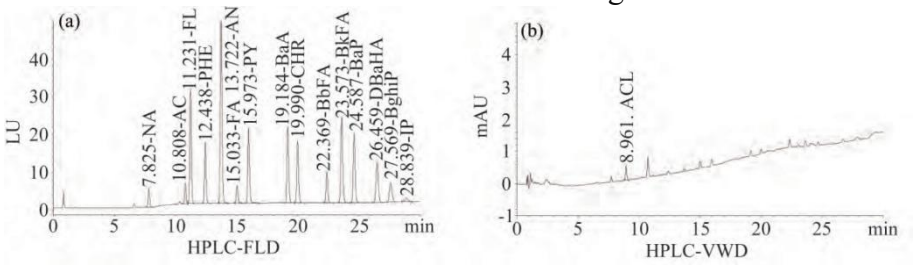

Figure 1 Chromatograms of 16 PAHS with concentration of 20 Ng/mL by HPLC-FLD(a)/VWD(b)

Table 2 Detection wavelength program of pahs

RT/min	E_X/nm	E_M/nm	PAH
0.00	265	325	NA, AC, FL
11.90	252	365	PHE
13.00	250	402	AN
14.40	280	460	FA
15.50	235	390	PY
17.50	270	390	BaA, CHR
21.50	280	430	Bbfa, bkfa, bap
25.50	284	395	Dbaha
26.90	290	435	Bghip
28.10	234	507	IP

2.2 Selection of purification column

Because aquatic products are rich in impurities such as lipids, impurities interfere seriously in the determination of PAHs content. The substrate noise is high. Therefore, silica gel is needed. Florisil. C18 purification column or MIP PAHs special column purification treatment [11]. Wang Wei et al. [10] compared C18. The purification effect of silica gel and Florisil purification column, in which the adsorption rate and elution rate of C18 column for PAHs are more than 99.5% and 78.7% respectively. MIPS pahs, a molecularly imprinted column, uses MIPS as an adsorbent

in solid phase extraction, which can specifically adsorb PAHs to solvents. Ions. Strong resistance to acid and alkali. According to the research of Li Cheng et al. [12], mips PAHs column is aligned to the middle ring. High cyclic PAHs have strong selective adsorption capacity and low matrix effect. The recovery rate of matrix standard addition is between 58% and 121%. Therefore, under the premise of Florisil column purification, the purification effect of C18 column and MIP PAHs on spiked samples was compared in this experiment.

The experiment took Sinonovacula constricta as the matrix, extracted with acetonitrile, purified by Florisil column, and then purified by C18 column and MIP PAHs column respectively to investigate the recovery rate of PAHs and the effect of impurity removal. According to table 3, after florisil-c$_1$8 column purification, the measured value of acenaphthene in the spiked group was 177.06 ng/mL lower than that in the blank group, that is, ACL was not detected. And there are many impurities in the sample, resulting in the interference of impurity peaks with the qualitative and quantitative analysis of pahs. However, after treatment with Florisil MIP pahs, the recovery rate of PAHs was between 60.2% and 91.6% (Table 3), which basically met the detection requirements. Moreover, Florisil MIP PAHs column has good impurity removal effect, and the interference peak is reduced, which is convenient for qualitative and quantitative [13]. Compared with MIP PAHs treatment [11], Florisil pretreatment helps to improve the recovery rate of MIP PAHs for naphthalene and other low ring PAHs in the sample and effectively remove impurities in shellfish samples. It may be that Florisil column pretreatment reduces the impurity content and increases the relative concentration of pahs, thereby increasing the adsorption capacity of MIP PAHs column for low ring pahs.

Table 3 Recoveries of PAHs on C18 And MIP-pahs purification column(n = 3)

PAH	Added/ (ng/mL)	C18		MIP-PAHs	
		Recovery /%	RSD /%	Recovery /%	RSD /%
NA	10.0	86.7	3.5	91.1	2.4
ACL	10.0	-	-	81.5	0.87
AC	10.0	83.6	1.5	60.2	4.1
FL	10.0	100.3	1.5	70.3	2.1
PHE	10.0	133.9	0.68	74.5	2.0
AN	10.0	97.6	0.56	82.3	4.3
FA	10.0	114.9	4.8	73.8	4.8
PY	10.0	149.8	5.1	80.1	5.6
Baa	10.0	81.0	2.0	86.8	0.77
CHR	10.0	81.2	1.01	81.7	0.78
Bbfa	10.0	84.8	0.86	89.1	1.2
Bkfa	10.0	74.5	0.74	75.4	5.8
Bap	10.0	80.5	1.6	81.4	0.52
Dbaha	10.0	84.9	1.1	89.9	0.43
Bghip	10.0	88.4	5.5	88.4	2.9
IP	10.0	88.6	4.0	91.6	2.5

2.3 Optimization of concentration conditions

Spinning vacuum. Concentration conditions such as nitrogen blowing rate and heating temperature will affect the recovery of pahs. According to Deng Xiaoyan et al. [14], fluorene. Phenanthrene and anthracene have low boiling points. The saturated vapor pressure is high, which is easy to be transferred to the air with the rapid flow of N_2 during nitrogen blowing, thus reducing the recovery rate. It is reported that the PAHs sample solution can be vacuum spinning combined with nitrogen blowing to shorten the concentration time and reduce the loss [15]. It was found that after direct nitrogen blowing treatment of pure standard solution, the loss rate of partial PAHs such as dibenzo [a, h]

anthracene and indene benzene [1, 2, 3-cd] pyrene was as high as 15%. It can be seen that slow nitrogen blowing increased the loss due to the extension of concentration time. In contrast, the vacuum rotary steamer can effectively shorten the concentration time under the conditions of low vacuum and low temperature water bath. However, when its concentration volume is less than 2 mL, it will aggravate the volatilization of pahs. Therefore, this experiment adopts the method of rotary evaporation combined with nitrogen blowing, that is, vacuum rotation (water bath temperature 35℃, vacuum degree ≤ 280 mbar, left about 5 mL, adjust vacuum degree ≤ 130 mbar, low-speed rotation) to about 2 mL, and then combined with nitrogen blowing (rate about 6 mL/min, heating temperature 35℃), condense the extract to about 0.8 mL, and then fix the volume. The recoveries of 16 PAHs were above 94.1% and RSDS were below 3.3%, which was better than direct nitrogen blowing or vacuum spinning.

2.4 Linear relationship of analysis method

According to the "1.4" method, PAHs of a series of mixed standard working solutions of 1, 5, 10, 20, 50 ng/mL were determined, and the peak area and the mass concentration of each component were plotted to obtain the corresponding regression equation and correlation coefficient of each component of pahs. Under this chromatographic detection condition, the linearity of 16 PAHs series standard working solutions is good, and the correlation coefficient (R^2) is above 0.9997, which can meet the requirements of quantitative analysis (Table 4).

2.5 Sensitivity and recovery of the method

In 5.0 g thick-shelled mussel matrix, 3 levels of 0.5, 1.5 and 2.0 μg/kg were added, respectively, and the sample preparation was performed according to the "1.3" method, and the "1.4" method was used to calculate the corresponding spike recovery. The experimental results showed that when the level of 0.5 μg/kg was added to the samples, the recoveries of 16 PAHs ranged from 58.1% to 89.6%, and the RSDs ranged from 0.23% to 11%. After adding a level of 1.5 μg/kg to the samples, the recovery of PAHs was in the range of 60.6%-88.1%, and the RSD was 1.3%-13%. In contrast, after adding 2.0 μg/kg to the sample, the corresponding recoveries of PAHs components increased, ranging from 72.3% to 98.0%, and the RSD was 1.5% to 14%. In addition, 0.5 μg/kg of PAHs mixed standard was added to 5.0 g thick-shelled mussel matrix, and six parallel experiments were carried out. and recoveries meet the requirements of trace analysis. Therefore, this method can meet the requirements of method sensitivity and recovery rate in the trace detection of shellfish products.

Table 4 Regression equations of 16 pahs

PAHs	Linear range/(ng/mL)	Regression equation	Correlation(R^2)
NA	1~50	$y = 1.18x + 2.47$	0.9997
ACL	1~50	$y = 0.16x + 0.028$	0.9999
AC	1~50	$y = 1.92x + 0.29$	0.9998
FL	1~50	$y = 11.2x + 3.38$	0.9999
PHE	1~50	$y = 5.94x + 2.77$	0.9999
AN	1~50	$y = 22.94x + 6.87$	0.9998
FA	1~50	$y = 1.82x + 0.50$	0.9999

PY	1~50	$y = 7.23x + 2.40$	0.9998
BaA	1~50	$y = 8.18x + 1.61$	0.9999
CHR	1~50	$y = 6.79x + 1.64$	0.9999
BbFA	1~50	$y = 3.46x + 0.46$	0.9999
BkFA	1~50	$y = 10.43x + 2.09$	0.9999
BaP	1~50	$y = 8.73x + 0.89$	0.9999
DbahA	1~50	$y = 5.71x + 0.069$	0.9999
BghiP	1~50	$y = 2.86x + 0.30$	0.9999
IP	1~50	$y = 0.69x + 0.14$	0.9999

2.6 Determination of different shellfish samples

Using the method established in this experiment, the levels of 2.0 μg/kg were added to 4 different shellfish matrices respectively, and the PAHs content in the blank matrix group was subtracted from the measured spiked value to calculate the recovery of 16 PAHs in the 4 shellfish samples. rate and RSD. Among them, the recoveries of 16 PAHs in the thick-shelled mussel samples ranged from 72.3% to 98.0%, and the RSDs ranged from 1.5% to 14%; the recoveries of PAHs in the constricted clams samples ranged from 60.2% to 91.6%, and the RSDs ranged from 0.43 to 0.43%. The recovery rate of PAHs in oyster samples was in the range of 61.9%-85.6%, and the RSD was in the range of 0.99%-15%; the recovery rate of PAHs in the clam samples was in the range of 65.8%-107.3%, and the RSD was in the range of 65.8%-107.3%. Between 3.0% and 14%.

3 Conclusion

In this study, acetonitrile water extraction was established. Florisil MIP PAHs column purification. Vacuum rotary evaporation - nitrogen blowing concentration. High performance liquid chromatography Ultraviolet/fluorescence detection combined with external standard quantitative analysis method is used to detect PAHs in shellfish samples. Experiments show that compared with florisi-$c_1$8 column, Florisil MIP PAHs column can effectively remove protein from shellfish samples. Impurities such as lipids interfere, reduce matrix effect, and contribute to the qualitative and quantitative analysis of pahs; compared with direct nitrogen blowing, the concentration method of vacuum spinning combined with nitrogen blowing can significantly shorten the time and reduce the loss of pahs. The results show that the method has good repeatability, strong operability and sensitivity. The recovery and precision meet the requirements of analysis and detection, and are suitable for the analysis and determination of trace PAHs in batch shellfish samples.

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