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The rapid determination of PAHs in foods using ultra high performance liquid chromatography

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UHPLC를 이용한 식품 중의 PAHs 신속분석법

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Abstract: Using a Hitachi LaChrom Ultra 2000U, a reverse phase ultra high performance liquid chromatography (u-HPLC) method was developed for the rapid quantification of 14 PAHs in foods. The proposed method for PAH analysis is based on solid phase extraction (SPE) cartridges; the determination was carried out by u-HPLC with fluorimetric detection. The method was very sensitive; PAH concentration levels were in a low μg/kg range and could be detected and quantified. Six samples of food were analyzed. Among PAHs, PHE was found in most of samples, the concentration ranging from 2.5 to 19.9 μg/kg. The contents of benzo[c]fluorine (BCL), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF) were low at the 'μg/kg' level or were less than LOD.

요 약: Hitachi LaChromUltra 2000U를 사용하여 역상의 초고속액체크로마토그래피 방법으로 식품 중의 PAHs 14종을 신속하게 분석하는 방법을 개발하였다. PAHs분석을 위한 방법은 solid phase extraction (SPE) 법에 의한 정제 후 u-HPLC를 사용하여 형광검출기로 분석하였다. 이 분석법은 감도가 매우 좋았으며, 식품 중의 PAHs를 μg/kg수준에서 분석이 가능하였다. 6개의 시료를 분석한 결과 모든 시료에서 PHE가 검출되었고, 그 함량은 2.5~19.9 μg/kg이었다. Benzo[c]fluorine (BCL), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF) 이 μg/kg이하의 농도에서 검출되었으나 그 농도는 모두 정량한계 이하이었다.

Key words : UHPLC, rapid analysis, PAHs

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are present in some petroleum products, coal tar, cigarette smoke, gasoline and diesel engine exhausts. Large amounts of PAHs deriving from incomplete combustion of organic matter are continuously released into the atmosphere from natural and anthropogenic sources. Through atmospheric fallout, these apolar substances can contaminate crops and are easily transferred to the final products, especially when the matrix has a lipidic nature (e.g. vegetable oil).[1-3] Seafood is a notable example of the various routes by which human foods may become contaminated by PAHs. PAHs enter the marine environmental from a variety of sources, including petroleum pollution and fallout from air pollution, and effluents from industries, sewage treatment plants and creosoted wharves. The practice of drying the meat before the oil extraction by the direct contact with combustion gases could greatly increase the PAH content.[1,4,5]

Food authorities from different countries all over the world have recommended different maximum residue limits. Most of these limits have been related to the sum of heavy (5- and 6-nuclear) PAHs and benzo[a]anthracene. In Spain, Italy and Canada, the limit of 3~5 μg/kg has been recommended.[1,6] In Germany, the limit of 5 μg/kg for the sum of heavy PAHs and 1 μg/kg for benzo[a]pyrene (BaP) have been recommended. Korea has a regulation for the level of BaP for edible oils and fats as 2.0 μg/kg. S. Moret *et al*. reported on the concentration of PAHs in vegetable oils from canned foods.[3] Of all the oil samples from vegetable products, 15% exceeded the 2 μg/kg level for BaP. The highest PAH concentration was found in the oil from a grilled mushroom sample, which contained 11.3 μg/kg of BaP and 47.1 μg/kg of total heavy PAH.

In the past decades, many methods have been developed to isolate, separate and quantitatively analyze the various PAHs in foods.[7-9] The most common methods for the isolation of PAHs from foods usually involved saponification of lipids by methanolic KOH followed by liquid-liquid partition and liquid-solid chromatography. The soxhlet extraction of PAHs, followed by the purification with a solid phase extraction (SPE) cartridge, was reported to remove more impurities than the sonification method.[10-14]

In this study, we tried to establish a rapid method to determine the concentration of PAHs in foods using ultra high performance liquid chromatography (u-HPLC) coupled with fluorescence detection. For the rapid analysis, rapid sample preparation and rapid separation are the two important aspects considered. For the rapid sample preparation, we followed a simple purification method to remove the hydrophobic fraction using an SPE cartridge. For the rapid separation in the chromatography, we used a short column (100 mm in length, 4.6 mm in diameter and 3.0 μm in pore size) to reduce the total running time.

2. Experimental

2.1. Materials and reagents

The PAHs phenanthrene (PHE), anthracene (ANT), benzo[e]fluorine (BcL), pyrene (PYR), benzo[a]anthracene (BaA), Chrysene (CHR), Benzo[k]-fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a] pyrene (BaP), dibenzo [a,l]pyrene (DIP), dibenzo[a,h] anthracene (DhA), benzo [g,h,i]perylene (BgP), dibenzo [a,e]pyrene (DeP) and dibenzo [a,i]pyrene (DiP) (Dr. Ehrenstorfer, Germany) were used. Stock standard methanolic solutions of PAHs at concentration levels of 100~500 μg/kg were prepared. These solutions were stored in glass bottles at 4 °C. Working PAH mixtures were prepared by diluting the above stock standard solutions with acetonitrile according to their sensitivities to fluorescence detection. HPLC grade hexane, dichloromethane (DCM) and acetonitrile (Aldrich, Steinheim, Germany) were used. The other solvents and chemical reagents were also HPLC grade. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

For the oil samples, 100 mg of sesame were dissolved with 1 mL of *n*-hexane in a 4 mL vial; then, 1 mL of the sample solution was loaded onto a

5 g silica SPE cartridge (LC-Silica Packing 20 mL, 5 g, Supelco, Bellefonte, PA, USA) previously washed with 20 mL of dichloromethane and conditioned with 20 mL of *n*-hexane. PAHs were eluted with the given amount of *n*-hexane-dichloromethane. First, 5 mL of eluate was discharged and the following 20~25 mL fraction, containing the PAH fraction, was collected in a conical vial. The flow rate was adjusted to about 1 drop per second.

The collected fraction was concentrated to about 20~30 μL of volume under a nitrogen stream, allowing the residual solvent to evaporate spontaneously at room temperature in order to minimize volatile PAH losses. The residue was dissolved in 1 mL of acetonitrile and filtered through a 0.20 μm syringe filter into a sample vial for HPLC injection.

An alkali digestion method was applied to the ham and crab samples. Two grams of meat were put into a 25 mL vial and 10 mL of 1 M ethanolic KOH was added. Then, the solution was gently refluxed for 30 min at 80 °C and extracted with 5 mL of *n*-hexane. 1 g of Na_2SO_4 and 1 g of silica was added, and the sample was shaken for 10 min. The sample solution was concentrated to approximately 1 mL on a rotary evaporator. One milliter of solution was filtrated through a 0.20 μm syringe filter into a vial for HPLC injection.

2.3. Chromatography

Two different HPLC systems were used. The conventional HPLC (c-HPLC) system consisted of a SHISEIDO HPLC apparatus (Shiseido, Tokyo, Japan), which included the following: an eluent reservoir; HPLC pump (SP3201); an autoinjection system (SP3023) of 20 μL injection at a fixed volume; fluorescence detector (SP3213).

The ultra-HPLC system consisted of LaChromUltra L-2000 U Series apparatus (Hitachi-High Technologies Corporation, Japan), which included the following: an eluant reservoir; HPLC pump (L-2160U); an autoinjection system (L-2200U) of 5 μL injection at a fixed volume; Fluorescence Detector (L-2485U). Three different columns were used to evaluate their performance: LaChromUltra C_{18} (2 mm i.d.×50 mm L, 2 μm, Hitachi-High Technologies Corporation, Japan); SUPELCOSIL LC_PAH (250 mm×3.0 mm, 5 μm, Supelco, Bellefonte, PA, USA); Agilent Eclipse_PAH (4.6×100 mm, 1.8 μm, Agilent Technology, USA). Fluorescence detection was performed with a program that had two excitation and emission wavelength pairs: λ_{ex}=270 nm/λ_{em}=390 nm for PHE, ANT, BcL, PYR, BaA, CHR; λ_{ex}=290 nm/λ_{em}=430 nm for BbF, BkF, BaP, DlP, DhA, BgP, DeP, and DiP.

The gradient was prepared by mixing water (solvent A) and acetonitrile (solvent B). The gradient profile for the separation of the PAHs by u-HPLC was 40% A-60% B (0 min), which was held for 0.5 min. Subsequently, the gradient profile changed linearly to 100% B in 5.0 min, which was held for 10.5 min and returned to 60% B in 0.5 min. The flow rate of u-HPLC was 1.2 mL/min and the temperature of the analytical column was maintained at 30 °C. The gradient profile for the separation of the PAHs by c-HPLC was 25% A-75% B (0 min), which was held for 8 min. Subsequently, it changed linearly to 100% B in 8 min, which was held for 15 min and returned to 75% B in 1 min. The flow rate of c-HPLC was 1.0 mL/min, and the analytical column was maintained at 30 °C.

2.4. Linearity

Calibration graphs for u-HPLC were based on peak area prepared by injecting 5 μL of PAH solutions at six concentration levels in the range of 0.1-50 μg/kg, which were prepared through the dilution of PAH stock solutions with acetonitrile.

2.5. Precision and accuracy

The reproducibility of the proposed method was expressed by the relative standard deviation (% RSD). Analysis of sesame oil containing PAHs was performed in triplicate. To evaluate the intra-day precision and accuracy, QC samples (n = 6) at low, middle and high concentrations were extracted in one batch. For the assay of the inter-day precision and accuracy, three consecutive batches of QC samples were made by the same procedure on three different days. Each

day, a new calibration curve was constructed when the samples were extracted. The precision and the accuracy were reported as the relative standard deviation (% RSD).

2.6. Recovery, LOD and LOQ

The recovery of the standard added to the assay samples was calculated from:

$$\text{Recovery, \%} = [(C_t - C_u) / C_a] \times 100$$

Where C_t is the total concentration of PAHs found, C_u is the concentration of PAHs present in the original sesame oil, and C_a is the concentration of the pure PAHs added to the original sesame oil. The detection limit was also studied. A calibration solution with a low concentration was analyzed by the present method. The limit of detection (LOD) and limit of quantitation (LOQ) values were estimated at an SD/b ratio of 3 and 10, where SD and b stand for the standard deviation of the intercept and slope of the regression line, respectively. To study the accuracy of the method, recovery experiments were performed using the standard addition method. Triplicate analyses of PAHs-added sesame oil were performed.

3. Results and Discussion

3.1. Evaluation of LC columns

Most method for PAH concentration determination involved sample preparation followed by HPLC on a reversed phase column (C_{18}) and spectrofluorometric detection. Recently, an ultra high performance liquid chromatography (u-HPLC) method, coupled to a fluorescence detector and a mass spectrometer, had been adopted for food and biological analysis because of its rapid analysis and excellent separation.[15-16] Because u-HPLC uses a higher pressure and shorter column than c-HPLC, peak dispersion was minimized and the method provided improved speed, resolution and sensitivity. u-HPLC delivers faster analysis, higher resolution, and increased sensitivity without decreasing the quality of separation. Moreover, the u-HPLC method is economical and environmentally friendly because of the extremely rapid analysis. In addition to the fast analysis, the consumption of solvent for mobile phase can be reduced up to 5 to 10 fold, compared with the c-HPLC method.

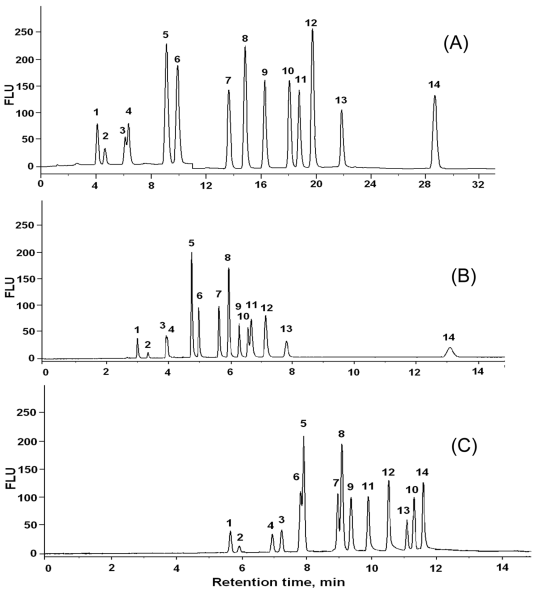

Fig. 1. Typical HPLC/FLD chromatograms of 14 PAHs : (A) a standard mixture of 14 PAHs using c-HPLC (Supleco-LC_PAH 250 mm×30 mm, 5 μm) at 10~50 μg/kg of PAHs; (B) a standard mixture of 14 PAHs using u-HPLC (Eclipse, PAH 100 mm×4.6 mm, 1.8 im) at 10~50 μg/kg of PAHs; (C) a standard mixture of 14 PAHs using u-HPLC (Hitachi C_{18}, 100 mm ×4.6 mm, 3 μm) at 10~50 μg/kg of PAHs. Chromatographic conditions are described in "Experimental". Peaks: 1, PHE; 2, ANT; 3, BcL; 4, PYR; 5, BaA; 6, CHR; 7, BbF; 8, BkF; 9, BaP; 10, DIP; 11, DhA; 12, BgP; 13, DeP; 14, DiP.

In this study, three different columns of different dimensions were investigated for the analysis of 14 PAHs by high performance liquid chromatography coupled to fluorescence detection. *Fig.* 1 shows the typical chromatograms of the comparison result of analyzing 14 PAH standards using c-HPLC and u-HPLC in the optimized conditions of each instrument. Optimization of the mobile phase led to a satisfactory resolution for all three columns.

However, because the use of the longer column (250 mm×30 mm, 5 μm in pore size) to obtain better separation by c-HPLC resulted in a retention time of PAHs by c-HPLC (about 32 min) that was twice as long as that of the u-HPLC (about 17 min), the

shorter column of u-HPLC (100 mm×4.6 mm, 1.8 μm in pore size) was used. The resolution of PAHs by the u-HPLC was sufficient to separate 14 PAHs for the quantification as illustrated in *Fig.* 1(C), although the total running time was finished within 13 minutes.

3.2. Refining processing

We evaluated the effects of the clean-up procedure on the recovery and separation. First, we injected the sample without any purification procedures to confirm the impurities in the sesame oil. Many impurities in the oil were eluted at the beginning of the chromatogram. Because a few PAHs, including PHE, ANT, PYR, and BcL, were co-eluted with impurities, quantification of PAHs in the sesame oil could not be satisfactorily achieved without a clean-up procedure.

To remove impurities, we employed the SPE cartridge clean-up procedure, as many papers had already suggested. With the clean-up procedure, many of the impurities eluted in the beginning of chromatogram were successfully removed and quantification could be achieved.

3.3. Linearity, LOD and LOQ

To determine the linearity, 6 different concentrations of PAH standards were used in a working range from 0.1 to 50.0 μg/mL of the 14 PAHs for a high performance liquid chromatographic method. Each solution was injected 3 times and the average values of triplicate analysis are represented. Regression analysis revealed a good correlation coefficient ($r^2 > 0.99$), as shown in *Table* 1.

For the determination of the LOD and LOQ of individual PAHs, we calculated the sensitivity using the 6 concentrations of the standard described above. The results are represented in *Table* 1. The LOD ranged from 0.10 μg/kg for CHR to 4.33 μg/kg for ANT. LOQ ranged from 0.29 μg/kg for CHR to

Table 1. Linearity and LOD/LOQ of PAHs

Analytes	Linear range μg/kg	Correlation Coefficient (r^2)	LOD μg/kg	LOQ μg/kg
PHE	0.50-50.00	0.9998	0.62	1.88
ANT	0.50-50.00	0.9953	4.33	13.12
BcL	0.30-30.00	0.9996	0.47	1.42
PYR	0.10-10.00	0.9994	0.22	0.65
BaA	0.20-20.00	0.9999	0.12	0.37
CHR	0.20-20.00	0.9999	0.10	0.29
BbF	0.30-30.00	0.9999	0.24	0.73
BkF	0.10-10.00	0.9993	0.22	0.68
BaP	0.10-10.00	0.9991	0.30	0.91
DlP	0.30-30.00	0.9998	0.24	0.73
DhA	0.30-30.00	0.9999	0.19	0.57
BgP	0.50-50.00	0.9999	0.32	0.98
DeP	0.30-30.00	0.9998	0.21	0.63
DiP	0.30-30.00	0.9990	0.57	1.73

Table 2. Precision data[a] of PAHs in sesame

Analytes	Amount added (μg/kg)	Intra-day RSD, %	Inter-day RSD, %
PHE	1	2.0	4.4
	5	1.2	2.7
ANT	2	1.1	0.8
	10	0.1	1.9
BcL	5	7.7	1.5
	20	0.4	3.4
PYR	3	3.6	5.9
	15	0.7	7
BaA	2	5.4	4.4
	10	2.8	2.1
CHR	2	2.0	1.5
	10	0.4	1.9
BbF	3	10.9	7.2
	15	0.3	3.1
BkF	1	1.6	1.2
	5	1.1	3.9
BaP	1	0.3	1.8
	5	0.6	3.1
DlP	3	0.8	3.9
	15	1.3	4.4
DhA	3	1.9	6.1
	15	1.4	3.8
BgP	5	6.4	8.9
	20	0.9	4.4
DeP	3	1.2	4.2
	15	2.0	4.8
DiP	3	1.3	3.9
	15	0.4	2.7

[a]Amount of sesame oil taken = 0.1 g; values represent the mean of intra-day (n = 6) and inter-day (n = 3).

13.12 μg/kg for ANT.

3.4. Precision and accuracy

We compared the precision and accuracy data of the PAHs at two concentrations for u-HPLC analysis. Intra-day ($n = 6$) and inter-day ($n = 3$) repeatability tests for precision were performed on the 14 PAHs in sesame oil. The relative standard deviations (RSDs) for intra-day and inter-day repeatability are represented in *Table* 2. The RSDs for the intra-day and inter-day repeatability were less than 10.9% and 8.9%, respectively.

To study the accuracy of the method, recovery experiments were carried out using a standard addition method. To enhance the accuracy of the proposed method, the nature and volume of the eluent were also studied; hexane-dichloromethane (70:30, v/v) and hexane-dichloromethane (95:5, v/v) were tested for the elution of 14 PAHs using 20 mL of solvent. The average of recovery for the 30% DCM was 62.6%, however, those of 5% DCM followed by 20 mL and 25 mL of eluting solvents were 92.2% and 90.7%, respectively. The highest recoveries were obtained using hexane-dichloromethane (95:5, v/v), the mean recovery was about 92.2%; therefore, this solvent was adopted as the eluent.

We also tested the effect of the volume of the eluting solvent on the recovery using either 20 mL or 25 mL of elution solvent. The accuracy data for the determination of PAHs in sesame oil with 5% DCM mixed with 95% hexane is given in *Table* 3. We found that there was no large difference between 20 mL and 25 mL of elution solvent applied to sesame oil samples, and that there was no significant difference found from *t*-test conducted to the amount of elution solvent. Most of the recovery ratios were higher than 70%, except the PHE, which ranged from 67.4 to 74.1%.

3.5. Analysis on real samples

The proposed method using u-HPLC was used to determine 14 PAHs in sesame and perilla oils, two crabs and two smoked hams. The chromatograms obtained are shown in *Fig.* 2. Retention times were used to identify the PAHs and the external calibration

Table 3. Accuracy data[a] for the determination of PAHs in sesame oil

Analytes	Amount added (μg/kg)	Recovery, %		
		30% DCM, 20 mL[b]	5% DCM, 20 mL[c]	5% DCM, 25 mL[d]
PHE	2.5	52.0	67.4	69.7
	12.5	69.1	68.5	71.3
	50	64.2	74.1	70.8
ANT	2.5	75.0	80.1	81.6
	12.5	68.0	76.7	83.1
	50	89.8	72.7	68.2
PYR	0.5	68.7	93.9	81.9
	2.5	62.3	78.2	74.7
	10	71.7	94.6	88.9
BcL	1.5	83.6	98.3	101.0
	7.5	74.8	86.2	86.0
	30	76.1	97.3	92.0
CHR	1	72.1	116.4	100.1
	5	67.3	95.1	93.6
	20	76.0	108.4	100.3
BaA	1	82.4	104.9	98.3
	5	72.8	94.7	96.2
	20	76.1	105.1	101.2
BbF	1.5	64.7	100.7	94.5
	7.5	64.7	95.8	96.1
	30	68.8	106.7	101.1
BkF	0.5	67.3	99.5	97.7
	2.5	64.0	93.9	96.6
	10	68.1	105.1	100.4
BaP	0.5	55.5	96.6	99.8
	2.5	55.3	92.0	94.9
	10	60.9	102.7	99.5
DlP	1.5	46.3	96.0	91.2
	7.5	44.8	92.4	96.8
	30	48.6	104.4	102.6
DhA	1.5	62.1	95.5	93.7
	7.5	59.4	92.2	94.4
	30	64.1	104.4	99.1
BgP	2.5	48.4	92.4	91.3
	12.5	47.3	92.9	95.7
	50	51.5	105.8	102.4
DeP	1.5	52.7	82.8	91.8
	7.5	49.7	88.1	93.5
	30	51.0	99.4	95.8
DiP	1.5	44.8	70.9	78.7
	7.5	43.5	70.5	76.2
	30	45.4	78.6	78.1

Table 3. Continued

Analytes	Amount added (μg/kg)	Recovery, %		
		30% DCM, 20 mL[b]	5% DCM, 20 mL[c]	5% DCM, 25 mL[d]
	Mean	62.6	92.2	90.7
	SD[e]	11.83	12.14	10.60
	RSD	18.89	13.17	11.68
t-test[f] (30% DCM - 5% DCM) = 0.333 (3.957) at P = 0.05				
t-test (5% DCM 5 mL - 5% DCM 25 mL) = 124.57 (3.957) at P = 0.05				

[a]Values represented the mean of triplicate analyses.
[b]*n*-hexane : DCM = 70:30 (v/v), volume of eluent = 20 mL.
[c]*n*-hexane : DCM = 95:5 (v/v), volume of eluent = 20 mL.
[d]*n*-hexane : DCM = 95:5 (v/v), volume of eluent = 25 mL.
[e]SD: standard deviation of mean of recoveries.
[f]Tabulated values of t and F at p = 0.05 are shown in parentheses

procedure was applied for quantification. *Table* 4 gives the results obtained from six samples. PHE was found in all the samples, the contents ranged from 8.2-20.0 μg/kg. The high concentration of PHE with respect to the other PAHs could be due to their high solubility in water.[17-18] The contents of BCL, PYR, BaA, CHR, BbF, BkF were very low, with concentrations at the μg/kg level or less than the LOD. The RSDs for the whole method ranged from 2~15% (n = 3). The results obtained were in accordance with published values, which were on the order of 1 μg/kg.[19-21]

4. Conclusions

A method for the determination of 14 PAHs in foods was proposed. This method was based on SPE cartridges and the determination was carried out by u-HPLC with fluorimetric detection. The method was more selective, rapid, and economical than those described in the literature for food samples, which were based on liquid-liquid extraction. The method

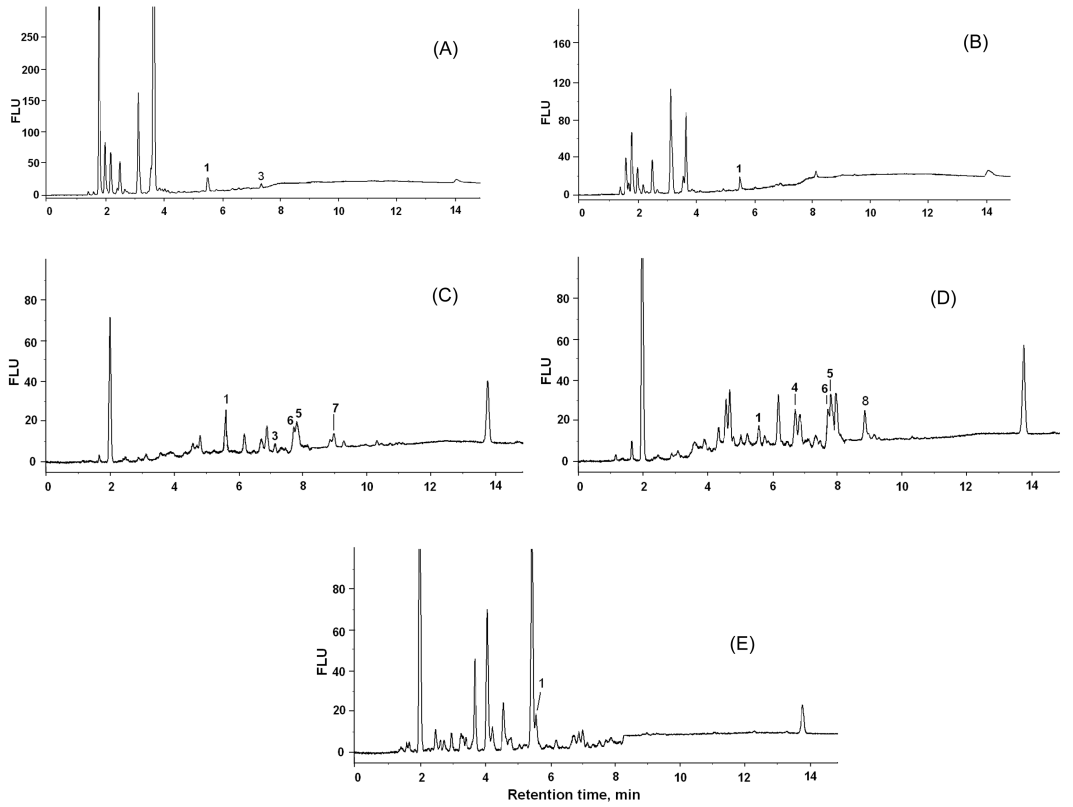

Fig. 2. u-HPLC/FLD chromatograms of PAHs: (A) sesame oil; (B) perilla oil; (C) Crab; (D) thin-shelled surf clam (E) smoked-ham A. The peak numbers are the same as those in *Fig.* 1.

Table 4. PAHs concentration[a] in various foods (μg/kg)

Analytes	Sesame oil	Perilla oil	Crab	Thin-shelled surf clam	Smoked-ham A	Smoked-ham B
PHE	2.5 ± 0.21	3.2 ± 0.12	19.9 ± 0.81	10.3 ± 0.07	8.2 ± 0.04	11.2 ± 0.12
ANT	ND[b]	ND	ND	ND	ND	ND
BcL	1.2 ± 0.03	ND	5.7 ± 0.06	ND	ND	ND
PYR	ND	ND	ND	1.3 ± 0.01	ND	ND
BaA	ND	ND	2.8 ± 0.01	0.7 ± 0.01	ND	ND
CHR	ND	ND	1.0 ± 0.02	1.01 ± 0.02	ND	ND
BbF	ND	ND	1.1 ± 0.01	ND	ND	ND
BkF	ND	ND	ND	0.8 ± 0.01	ND	ND
BaP	ND	ND	ND	ND	ND	ND
DlP	ND	ND	ND	ND	ND	ND
DhA	ND	ND	ND	ND	ND	ND
BgP	ND	ND	ND	ND	ND	ND
DeP	ND	ND	ND	ND	ND	ND
DiP	ND	ND	ND	ND	ND	ND

[a]Values represented the mean of triplicate analyses ± standard deviation.
[b]Not detected.

was very sensitive; PAH concentration levels in the μg/kg range could be detected and quantified. Six samples of food were analyzed. PHE was found in all samples; the concentration ranged from 2.5~19.9 μg/kg. The concentrations of BCL, PYR, BaA, CHR, BbF, and BkF were very low, with a range at μg/kg level or less than the LOD.

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