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Determination of polycyclic aromatic hydrocarbons in milk samples by high-performance liquid chromatography with fluorescence detection

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Abstract

This paper describes a high-performance liquid chromatographic (HPLC) method for the determination of polycyclic aromatic hydrocarbons (PAHs) in milk samples. The method involves a liquid–liquid extraction procedure after saponification of milk samples with sodium hydroxide. Reproducible determination with highly sensitive detection was attained by HPLC with fluorescence detection using 1,2-bis(9-anthryl)ethane as an internal standard. The detection limits of 12 kinds of PAHs ranged from 1.3 to 76 ng/kg milk at a signal/noise ratio of 3. By the proposed method, the presence of 12 and 11 kinds of PAHs could be confirmed in commercial milk and human milk samples, respectively. The average concentrations of total PAHs (mean±SD, μg/kg) were found to be 0.99±0.37 for commercial milk (n=14), 2.01±0.30 for infant formula (n=3) and 0.75±0.47 for human milk (n=51). High correlation coefficients between the concentrations of total PAHs and triglyceride were observed for commercial milk (r=0.659) and human milk (r=0.645).
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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) originate from the incomplete combustion or pyrolysis of organic matter such as fuel oils [1]. PAHs present in the environment are mainly due to their release from motor vehicles and various industrial sources [2,3] and are widely distributed as pollutants [4]. A large number of PAHs are known to have carcinogenic and/or mutagenic properties [5]. Recently, some PAHs are suspected to be endocrine disrupters [6,7] and much attention have been focussed on their possible biological effects on human health.

Human exposure to PAHs occurs mainly by inhalation of airborne particulates containing PAHs and intake of dietary products contaminated by PAHs [8,9]. After absorption in the body, PAHs are largely excreted in urine or feces as hydroxylated metabo-

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lites [10,11]. In addition, PAHs accumulate in adipose tissue because of their lipophilicity [12], and may be excreted in human milk. Even if the presence of contaminants in human milk is slight, it may affect the health of breast-fed infants since they are more sensitive than adults to these contaminants. From this point of view, a highly selective and sensitive method for determination of PAHs in milk samples is required.

For the determination of PAHs in human milk, a few works have been reported, by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [13] and by gas chromatography with mass spectrometry (GC–MS) [14]. However, their sensitivity and reliability were not enough to determine the trace levels of PAHs in human milk.

Our main object in this work was to establish a sensitive, reliable and selective analytical method for the determination of PAHs in milk samples. To meet these requirements, we developed an HPLC method with fluorescence detection following liquid–liquid extraction of PAHs after saponification of the samples. An internal standard (I.S.) was employed in the proposed method to obtain reproducible results. The proposed method was successfully applied to the elucidation of the species and the determination of PAHs which exist in these samples.

2. Experimental

2.1. Chemicals

Anthracene (A, 99.5% purity), fluoranthene (Fl, 97% purity), benzo[*a*]pyrene (BaP, 98% purity) and sodium hydroxide were obtained from Wako Pure Chemical Industries (Osaka, Japan). Phenanthrene (P, 98% purity), pyrene (Py, 97% purity) and benz[*a*]anthracene (BaA, 98% purity) from Tokyo Kasei Kogyo (Tokyo, Japan) were used. Chrysene (Ch, 95% purity) and benzo[*g,h,i*]perylene (BP, 98% purity) were purchased from Aldrich (Milwaukee, WI, USA). Benzo[*b*]fluoranthene (BbF, 98% purity), benzo[*k*]fluoranthene (BkF, 98% purity) and indeno[1,2,3-*cd*]pyrene (IP, 99% purity) were supplied from Supelco (Bellefonte, PA, USA). Dibenzo[*a,h*]anthracene (DahA, 97% purity) was purchased from Sigma (St. Louis, MO, USA). 1,2-Bis(9-anthryl)ethane as an I.S. was synthesized as reported previously [15]. Water was passed through a Pure Line WL 21 P system (Yamato Scientific, Tokyo, Japan). *n*-Hexane was used as received from Nacalai Tesque (Kyoto, Japan). Acetonitrile was of HPLC grade (Wako) and other chemicals were of analytical reagent grade.

2.2. HPLC system

The HPLC system consisted of a liquid chromatographic system LC module 1 (Waters, Milford, MA, USA), a 7125 injector (Rheodyne, Cotati, CA, USA) with a 20-μl sample loop, a column oven HTR-B (Waters), an RF-550 fluorescence detector (Shimadzu, Kyoto), and a Chromatocorder 12 integrator (System Instruments, Tokyo, Japan). Separation of PAHs was achieved by a Wakosil-PAHs column (250×4.6-mm I.D., particle size 5 μm, Wako) with a gradient elution program using solvent A (MeOH–H_2O, 8:2, v/v) and solvent B (CH_3CN). The gradient elution was programmed as follows: 10% B (0–4 min), 10% B to 95% B linearly (4–7 min), and 95% B (7–30 min). The flow-rate and the column temperature were set at 1.0 ml/min and 35 °C, respectively. Under these conditions, 12 PAHs and I.S. could be separated satisfactorily within 30 min. The detection wavelength program to determine individual PAHs is shown in Table 1.

2.3. Sample collection

Commercial milk (six samples of whole milk, three samples of skimmed milk, three samples of condensed milk and two samples of fortified milk)

Table 1
Detection wavelength program for measurement of PAHs

Time (min)	λ_{ex} (nm)	λ_{em} (nm)
0–8.4	248	360
8.4–9.8	254	400
9.8–11.2	360	462
11.2–15.0	337	392
15.0–22.5	284	398
22.5–23.5	364	500
23.5–end	250	420

and infant formula (three samples) used in this study were commercially available. The infant formula was prepared by dissolving in hot water according to the manufacture's recommendation. The human milk (51 samples) was collected twice, 1 week and 1 month after parturition from each mother. Milk samples were stored at −25 °C and thawed in a water bath at 37 °C for 5 min just prior to the analysis.

2.4. Sample preparation

Two grams each of the milk samples in a 10-ml reaction vial with a screw cap were saponificated with 4.0 ml of 0.4 *M* sodium hydroxide in EtOH–H_2O (9:1, v/v) at 60 °C for 30 min after addition of 100 μl of I.S. solution in CH_3CN (12.5 or 25.0 n*M*). The resultant solution was extracted twice with 2.0 ml of *n*-hexane. After combining the extracts, the *n*-hexane phase was evaporated to dryness and the residue dissolved in 100 μl of CH_3CN was passed through a membrane filter (0.45 μm). An aliquot of 20 μl of this solution was injected into the HPLC system.

2.5. Triglyceride assay in milk sample

Triglyceride concentration of milk samples as an indicator of milk fats was determined by using the Triglyceride E-Test Wako as follows: a milk sample was diluted 10 times with water and then aliquots of 10 μl of the diluted milk and 3.0 ml of color-producing reagent were added in a reaction vial successively. After incubation for 5 min at 37 °C, the color development was measured at 600 nm by a Shimadzu Model 265 FS spectrophotometer. A calibration curve was prepared with a series of standard aqueous solutions which are consisted of glycerin and triolein.

3. Results and discussions

3.1. Optimization of extraction conditions

To test the efficiency of extraction method, the extractability of PAHs through the entire procedure were determined using spiked milk which was prepared by shaking for 1 h after addition of standard PAHs solution.

In a preliminary study, a liquid–liquid extraction was examined by using *n*-hexane, cyclohexane and toluene as solvents. No significant differences in the extractability of standard PAHs spiked to commercial milk were observed in these solvents. Thus, *n*-hexane was chosen for further experiments. The effect of the number of repeated extractions on the extractability was examined with 2.0 ml each of *n*-hexane (Fig. 1). The maximum and constant extractability was almost attained for 2- or more-fold extraction: therefore, the number of extractions was set at two.

The concentration of sodium hydroxide in the saponification solution affected the extractability (Fig. 2). The maximum extractabilities were observed with sodium hydroxide concentrations in the range 0.3–0.6 *M*; 0.4 *M* was chosen.

The contents of EtOH in the saponification solution (ranging from 60 to 100%) also influenced the extractability (Fig. 3). Although better extractabilities were observed at 100% EtOH for most PAHs, the interfering peaks also increased simul-

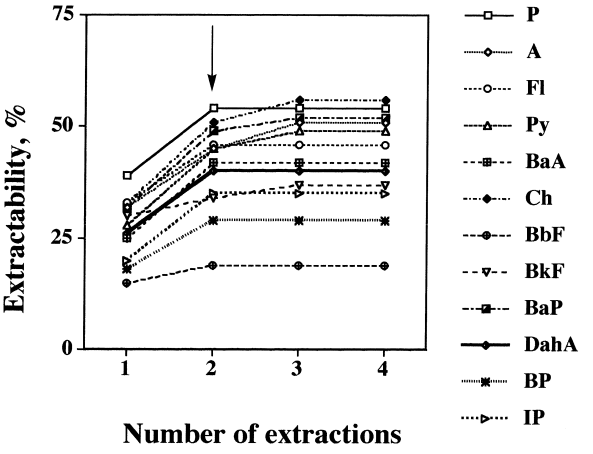

Fig. 1. Effect of the number of extractions on extractability. Sample concentration: P = 0.89 μg/kg, A = 0.09 μg/kg, Fl, Py = 1.01 μg/kg, BaA = 0.11 μg/kg, Ch = 1.14 μg/kg, BbF = 0.63 μg/kg, BkF, BaP = 0.06 μg/kg, DahA = 0.14 μg/kg, BP = 0.70 μg/kg, IP = 1.38 μg/kg; NaOH concentration, 0.2 *M*; saponification time, 120 min; saponification temperature, 60 °C; the other conditions are given in Section 2.

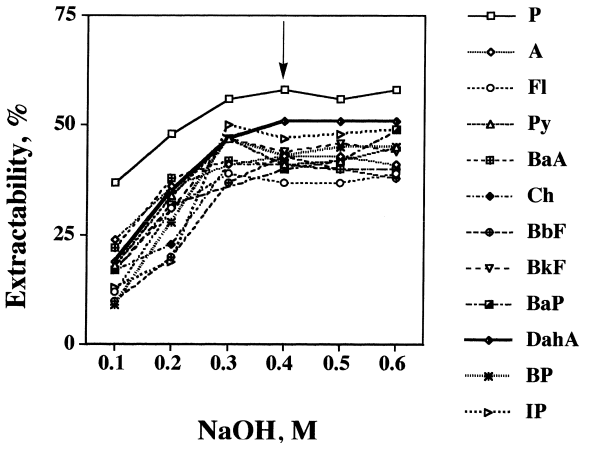

Fig. 2. Effect of NaOH concentration on extractability. Sample concentration: P = 0.89 μg/kg, A = 0.09 μg/kg, Fl, Py = 1.01 μg/kg, BaA = 0.11 μg/kg, Ch = 1.14 μg/kg, BbF = 0.63 μg/kg, BkF, BaP = 0.06 μg/kg, DahA = 0.14 μg/kg, BP = 0.70 μg/kg, IP = 1.38 μg/kg; saponification time, 120 min; saponification temperature, 60 °C; the other conditions are shown in Section 2.

taneously and the peak for P was unable to be confirmed. Therefore, 90% EtOH was chosen.

The effects of the saponification time and temperature on the extractability were examined (Fig. 4). Saponification temperature at 60 °C provided better results and thus was selected with saponification time for 30 min.

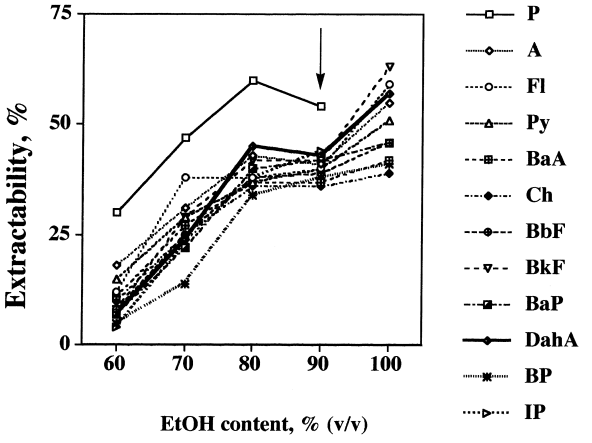

Fig. 3. Effect of EtOH concentration in the saponification solution on extractability. Sample concentration: P = 0.89 μg/kg, A = 0.09 μg/kg, Fl, Py = 1.01 μg/kg, BaA = 0.11 μg/kg, Ch = 1.14 μg/kg, BbF = 0.63 μg/kg, BkF, BaP = 0.06 μg/kg, DahA = 0.14 μg/kg, BP = 0.70 μg/kg, IP = 1.38 μg/kg; saponification time, 120 min; saponification temperature, 60 °C; the other conditions are shown in Section 2.

3.2. Calibration curves, detection limits and reproducibility

Calibration curves were independently prepared for commercial milk and human milk samples which were spiked with standard PAHs. Table 2 shows calibration curves, calibration ranges and detection limits obtained with spiked human milk. In both the samples, good linearities ($r \geq 0.992$) were obtained between the peak height ratios and concentrations. The limits of detection at signal/noise ratio (S/N) of 3 were in the range 1.3–76 ng/kg (commercial milk) and 1.3–35 ng/kg (human milk). The sensitivity of the proposed method for BaP was 100 times higher than that of GC–MS method [14] and 10 times higher than those of HPLC–UV [13] and HPLC-fluorescence methods for commercial milk [16]. Fig. 5 represents typical chromatograms obtained with milk samples and commercial milk spiked with standard PAHs.

The coefficient of variation (%, C.V.) in within-day precision studies ($n = 4$) ranged from 3.5 to 9.8% (Table 3). Recoveries, which were calculated by using observed and spiked concentrations for PAHs, ranged from 90 to 105% (Table 3), and the recovery of I.S. was 94%. Also, between-day precision studies ($n = 4$) gave satisfactory reproducibilities (6.9–10.2%) as with the within-day precision studies.

3.3. Determination of PAHs in milk samples

The proposed method was applied to determine PAHs in milk samples. The amount of PAHs in commercial milk, infant formula and human milk samples are shown in Table 4. The average concentrations (toxic equivalency factors adjusted concentration [15]) of total PAHs in commercial milk, infant formula and human milk were 0.99 (0.16), 2.01 (0.40) and 0.75 (0.09) μg/kg, respectively. The results obtained from commercial milk were in accordance with those in previous report except for BbF [17]. The difference of the values for BbF might be due to the difference of cattle-breeding sites and

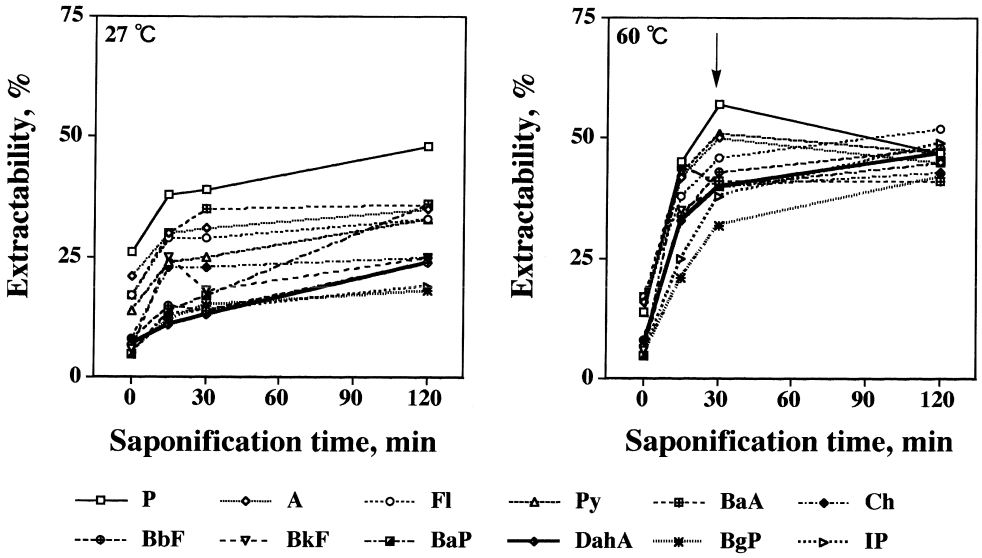

Fig. 4. Effects of saponification time and temperature on extractability. Sample concentration: P = 0.89 μg/kg, A = 0.09 μg/kg, Fl, Py = 1.01 μg/kg, BaA = 0.11 μg/kg, Ch = 1.14 μg/kg, BbF = 0.63 μg/kg, BkF, BaP = 0.06 μg/kg, DahA = 0.14 μg/kg, BP = 0.70 μg/kg, IP = 1.38 μg/kg; the other conditions are shown in Section 2.

processing sites of milk between UK and Japan. The infant formula contained higher levels of PAHs than commercial milk and human milk. For this reason, PAHs might be formed during drying processes where direct heating with fuel gas is employed [18,19].

3.4. Correlation between concentrations of PAHs and triglyceride in milk samples

It is thought that PAHs are incorporated in fats of milk owing to their lipophilic nature. Triglyceride is known to be main components of fats in commercial

Table 2
Calibration curves, calibration ranges and detection limits for spiked human milk

Compound	Calibration curve $y = (\text{slope} \pm \text{S.E.})x + (\text{intercept} \pm \text{S.E.})$	Correlation coefficient (r)	Calibration range (μg/kg milk)	Detection limit $S/N = 3$	
				(μg/kg milk)	(fmol/injection)
P	$y = (9.13 \pm 0.20)x + (0.19 \pm 0.06)$	0.997	0.04–1.78	0.033	74
A	$y = (40.34 \pm 0.88)x + (0.08 \pm 0.03)$	0.994	0.005–0.09	0.0045	10
Fl	$y = (0.85 \pm 0.02)x + (0.01 \pm 0.003)$	0.995	0.02–1.01	0.011	22
Py	$y = (2.60 \pm 0.04)x + (0.03 \pm 0.03)$	0.995	0.03–1.52	0.0086	17
BaA	$y = (15.75 \pm 0.12)x + (0.02 \pm 0.06)$	0.994	0.006–0.06	0.0020	3.5
Ch	$y = (0.97 \pm 0.01)x + (0.02 \pm 0.008)$	0.992	0.03–0.57	0.029	50
BbF	$y = (2.65 \pm 0.03)x + (0.08 \pm 0.005)$	0.994	0.04–1.89	0.011	17
BkF	$y = (22.01 \pm 0.37)x + (0.003 \pm 0.002)$	0.999	0.001–0.03	0.0013	2.0
BaP	$y = (26.24 \pm 0.45)x + (0.02 \pm 0.002)$	0.992	0.001–0.01	0.0013	2.0
DahA	$y = (4.89 \pm 0.07)x + (0.07 \pm 0.007)$	0.997	0.007–0.07	0.0070	10
BP	$y = (1.21 \pm 0.02)x + (0.01 \pm 0.006)$	0.995	0.04–0.35	0.035	50
IP	$y = (0.62 \pm 0.01)x + (0.01 \pm 0.001)$	0.999	0.01–0.28	0.014	20

x = concentration (μg/kg milk); y = peak height/I.S. peak height.

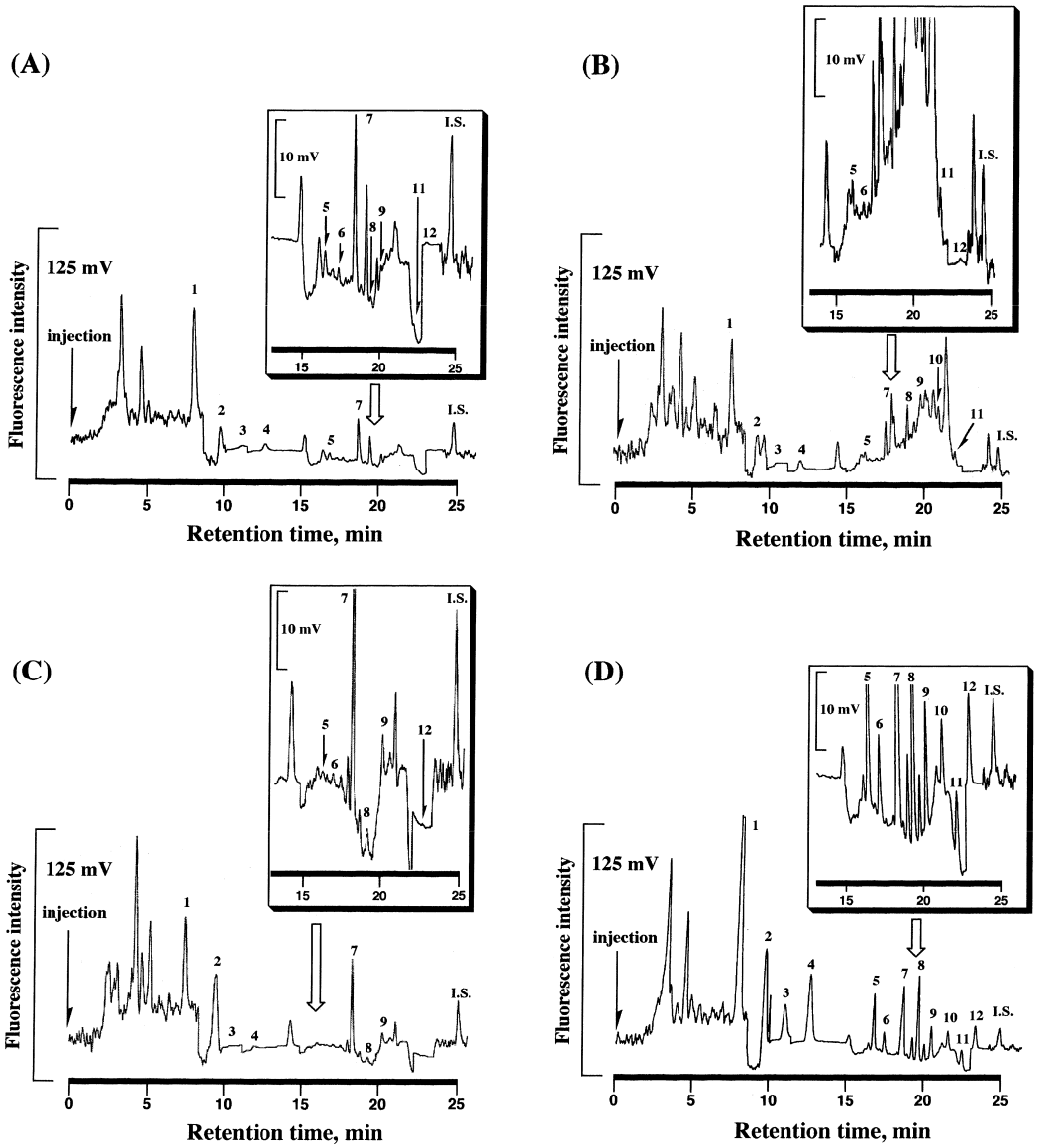

Fig. 5. Chromatograms of PAHs in (A) commercial milk, (B) infant formula, (C) human milk and (D) commercial milk spiked with standard PAHs. Peaks: (1) P; (2) A; (3) Fl; (4) Py; (5) BaA; (6) Ch; (7) BbF; (8) BkF; (9) BaP; (10) DahA; (11) BP; (12) IP. Spiked concentration: P = 0.09 μg/kg, A = 0.01 μg/kg, Fl = 0.4 μg/kg, Py = 0.2 μg/kg, BaA = 0.02 μg/kg, Ch = 0.23 μg/kg, BbF = 0.13 μg/kg, BkF = 0.01 μg/kg, BaP = 0.01 μg/kg, DahA = 0.03 μg/kg, BP = 0.14 μg/kg, IP = 0.28 μg/kg, I.S. = 0.24 μg/kg.

milk and human milk. Therefore, the correlation between concentrations of total PAHs and triglyceride in milk samples were investigated (Fig. 6). Good correlations were observed for commercial milk ($r = 0.659$, $n = 14$). This result indicates that the commercial milk that contain more triglyceride provide higher levels of PAHs. In the case of commercial milk, PAHs might be able to be reduced with the process of skimming. On the other hand, human milk showed a variation of triglyceride concentration at 4.82–48.98 mg/ml, which are in good agreement with the published data [20], and

Table 3
Recovery and precision of the method

Compound	Spiked concentration (μg/kg milk)	Within-day ($n=4$)		
		Found (μg/kg milk)	Recovery[a] (%)	C.V.[b] (%)
P	0.446	0.412	92	8.4
A	0.022	0.023	105	6.0
Fl	0.253	0.228	90	3.5
Py	0.379	0.362	96	9.3
BaA	0.014	0.014	100	7.0
Ch	0.142	0.130	91	8.4
BbF	0.473	0.443	94	6.2
BkF	0.006	0.006	100	9.8
BaP	0.006	0.006	100	8.3
DahA	0.070	0.070	100	6.7
BP	0.350	0.315	90	8.9
IP	0.280	0.292	104	7.2

[a] Expressed as [(mean observed concentration)/(spiked concentration)]×100.
[b] C.V., coefficient of variation.

were also correlated with total PAHs concentration ($r=0.645$, $n=51$).

4. Conclusions

This study provides a sensitive and reliable HPLC method for the fluorescence determination of PAHs in milk samples using a simple procedure for sample preparation. The detection limits of PAHs in milk samples ranged from 1.3 to 76 ng/kg. By using this method, total PAHs were detected at 0.23–2.01 and 0.19–2.15 μg/kg levels in commercial milk and human milk, respectively.

The proposed method should be applicable to the monitoring of PAHs in human milk for epidemiologi-

Table 4
Concentrations (μg/kg milk) of PAHs in milk samples

Compound	Concentration (μg/kg milk)					
	Commercial milk ($n=14$)[a]		Infant formula ($n=3$)[a]		Human milk ($n=51$)	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
P	0.27±0.09	0.09–0.48	0.40±0.10	0.30–0.49	0.25±0.16	0.04–0.99
A	0.01±0.01	N.D.[b]–0.02	0.02±0.01	0.01–0.03	0.005±0.007	N.D.–0.03
Fl	0.14±0.07	N.D.–0.25	0.19±0.07	0.12–0.25	0.02±0.03	N.D.–0.13
Py	0.11±0.07	N.D.–0.27	0.16±0.08	0.08–0.24	0.02±0.05	N.D.–0.29
BaA	0.02±0.02	N.D.–0.08	0.04±0.02	0.02–0.06	0.004±0.009	N.D.–0.04
Ch	0.10±0.07	N.D.–0.22	0.25±0.14	0.14–0.40	0.06±0.08	N.D.–0.32
BbF	0.35±0.20	0.13–0.63	0.36±0.16	0.20–0.51	0.41±0.26	0.01–1.23
BkF	0.02±0.01	N.D.–0.03	0.05±0.02	0.03–0.06	0.006±0.006	0.001–0.03
BaP	0.03±0.02	N.D.–0.06	0.05±0.02	0.03–0.06	0.002±0.003	N.D.–0.01
DahA	0.02±0.01	N.D.–0.04	0.06±0.04	0.02–0.10	0.007±0.012	N.D.–0.06
BP	Trace[c]	Trace	0.32±0.06	0.27–0.39	N.D.	N.D.
IP	0.01±0.01	N.D.–0.03	0.12±0.08	0.04–0.20	0.003±0.007	N.D.–0.03
Total PAHs	0.99±0.37	0.23–2.01	2.01±0.30	1.68–2.28	0.75±0.47	0.19–2.15

[a] Mean of duplicate measurement.
[b] N.D., not detectable.
[c] The value between 0.14 and 0.7 μg/kg.

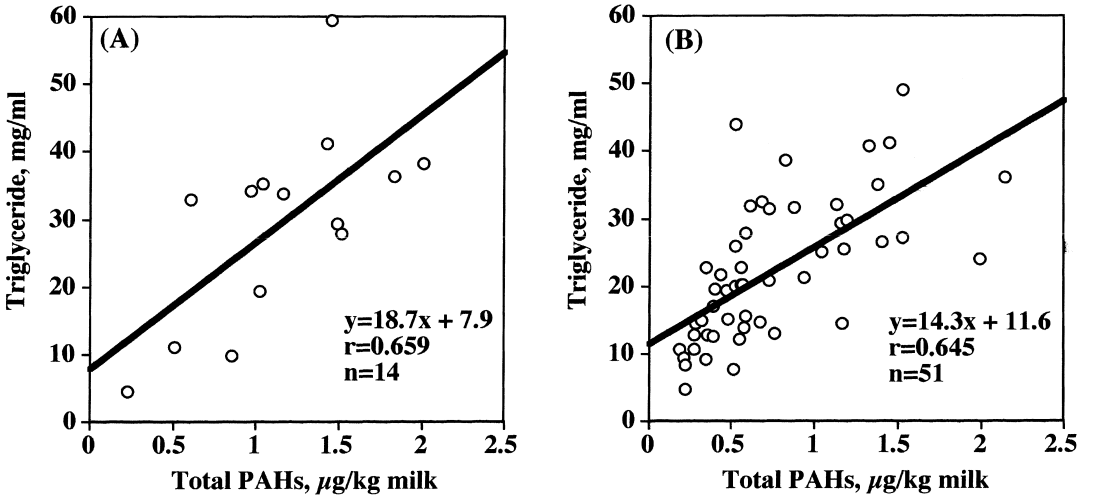

Fig. 6. Correlations between the concentrations of total PAHs and triglyceride in (A) commercial milk and (B) human milk.

cal and toxicological studies. In addition, this method might be useful for estimating the pollution of PAHs in commercial milk samples.

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