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Determination of naphthalene by competitive fluorescence immunoassay

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Abstract A reliable and sensitive competitive fluorescence immunoassay for the quantitative determination of naphthalene (NA) was developed. 2-naphthoxy acetic acid (NAA) was selected as the hapten of naphthalene. Active ester method (AEM) was used to couple the NAA to carrier proteins (bovine serum albumin) to form artificial immune antigen. Male New Zealand white rabbits were immunized with this antigen to obtain polyclonal antibodies, with which, a novel fluorescence immunoassay for detection of NA was described. Under best conditions, NA can be determined in the concentration range of 0.1–100 μg/L with a detection limit of 0.05 μg/L. The cross-reactivities of the anti-NA antibody to seven structurally related compounds were below 15%. Some environmental samples were analyzed with satisfactory results. It shows a good accuracy and suitability to analyze NA in environmental water.

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Introduction

Naphthalene (NA) is the simplest and most abundant of the polycyclic aromatic hydrocarbons (PAHs) present in gasoline and diesel fuels (Linsey et al. 1999) and is various used in the manufacture of dyes, plastics, leather tanning agents and many other products. However, NA is defined as a hazardous in environment by the US Environmental Protection Agency. It can damage or destroy red blood cells and cause hemolytic anemia if exposured to high concentration of NA (Lu et al. 2005). Due to it importance as soil and groundwater contaminants, attention has been paid towards the determination of it. Current official analytical methods for NA were based on gas chromatography (GC; Anjaneyulu et al. 2006; Hung et al. 1999) and High Performance Liquid Chromatography (HPLC; Díaz et al. 1999). In GC, methods of gas chromatography-mass spectrometry (Guehenneux et al. 2004; Pavón et al. 2007) gas chromatography with flame ionisation detection (Bianchi et al. 2005; Lehotay and Hromulakova 1997) have been reported. Method of fluorescence spectra (Tang et al. 1995) has also been reported as method for detecting

of NA. However, the complex sample treatment, including preconcentration and derivatization, can give misleading results of the detection. Therefore, the development of an inexpensive, fast, and sensitive method for NA is of primary interest. Immunological methods provide an opportunity to screen large sample volumes quickly and cost effectively (Yu et al. 2006; Zhang et al. 2007). But no fluorescence immunoassays for NA have been reported in the literature. Here, we propose a new method to analyze NA by using competitive fluoroimmunoassay (FIA) that was not reported previously. Fluorescence immunoassays based on selective antigen-antibody binding and fluorescence label reagents have gained increasing importance in recent years (Morita et al. 2005; Cao et al. 2003; Butler and Guilbault 2004; Nunnally 2005). Here, we propose a new sensitive and selective competitive fluorescence immunoassay for the determination of NA in water samples.

Experimental

Chemicals

Goat anti-rabbit IgG-FITC, *N*,*N*′-dimethylformamide, Sephadex G-25, *N*,*N*′-dicyclohedyicarbodiimid, *N*-hydroxysuccinimde were obtained from Sigma (Shanghai, China); Bovine serum albumin (BSA, MW 67000) and Ovalbumin (OVA, MW 45000) were purchased from Sino-American Biotechnology Co. (Shanghai, China). Freund's complete adjuvant (lanoline/mineral oil 1:4, and heat-killed mycobacterium tuberculosis 3 mg/kg each rabbit) and Freund's incomplete adjuvant (lanoline/mineral oil 1:4 were prepared in our laboratory. The stock solution of anti-NA antibody (100 μg/mL) was stored at 4°C and protected from light. NA was obtained from Shanghai Chemical Reagent Co. (Shanghai, China) and purified. Standard NA solution (1.0 mg/mL) was dissolved in ethanol and stored at 4°C. Buffers for the immunoassay procedure were prepared by routine methods. 2-naphthoxy acetic acid (NAA) was purchased from CCA (Changzhou) Biochemical Co. Ltd. (Jiangshu, China). Chemical reagents were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Immunochemicals were obtained from Sino-American Biotechnology Co. (Shanghai, China). Unless otherwise indicated, data presented correspond to the average of at least five well replicates. All chemicals were of analytical reagent grade, and double-distilled water was used throughout.

Apparatus

The apparatus were a TU-1900 ultraviolet/visible spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., NEXUS670 Fourier transform infrared ray spectroscopy (Nicolet Company), AV^{1}H-Nuclear Magnetic Resonance spectra (400 Hz, Bruket), QP-2010 Pyrolysis/Gas Chromatography/Mass Spectroscopy (Shimadzu Corporation), Flx-800 plate reader (BIO-TEX), 96-wells FIA plates (Gene Co Ltd.).

Preparation of polyclonal antibodies

Because the target NA is of a small molecular weight (MW128), it requires conjugation to carrier proteins in order to be immunogenic. NAA was purchased from CCA (Changzhou) Biochemical Co. Ltd. (Jiangshu, China) as the hapten. Then NAA was conjugated to BSA or OVA via amino diazotization linkage. The NA-BSA conjugate was used as an immunogen to immunize two female New Zealand white rabbits. The final serum was collected 3 months following the first immunization. The blood was collected

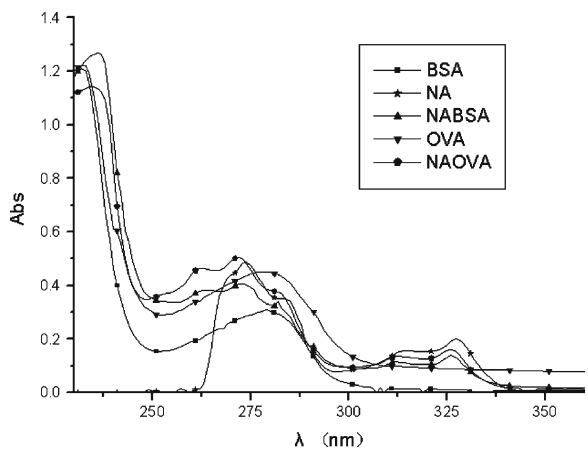


Fig. 1 UV Spectra of the NA hapten conjugates and carrier proteins

Table 1 Titers of antisera determined by agar diffusion test and indirect FIA

Titers	Boost						
	1st	2nd	3rd	4th	5th	6th	7th
Agar test	None	1:2	1:8	1:16	1:32	1:32	1:64
Indirect FIA	1:4,000	1:8,000	1:32,000	1:32,000	1:64,000	1:128,000	1:128,000

into the glass tube. The antiserum was obtained by centrifugation, and the immunoglobulin G (IgG) fraction of the antiserum was isolated by precipitation with saturated ammonium sulfate solution. After dialysis against phosphate-buffered saline (PBS), the purified IgG fractions were lyophilized aliquoted into vials and stored at −20°C until use.

Fluorescence immunoassay procedure

The indirect competitive FIA procedure was performed as follows: Each well of the 96-well microtiter plates was coated with 100 μL/well NA–OVA (2 μg/mL) in CBS (50 mM pH 9.6) and incubated overnight at 4°C. The plates were washed three times with 200 μL/well PBS with 0.05% Tween 20 (PBST) and were blocked by incubation with 0.5% OVA in PBST at 37°C for 1 h. After another washing step, serial dilution of the analyte standard in PBS was added, 50 μL/well. Followed by addition of 50 μL/well of purified antiserum previously diluted with PBST (1/4,000). After incubation at 37°C for 1 h, the plates were washed and 100 μL/well of a diluted goat antirabbit IgG-FITC (1:1000) was added. The mixture was incubated at 37°C for 1 h and then washed five times. The fluorescence intensity of each well was determined with the BIO-TEK microplate reader at λex485 nm, λem528 nm, and the fluorescence intensity difference between F and F0 was calculated, where F0 is the fluorescence intensity in the absence of labeled antibody. Standard curves were obtained by plotting the (F-F0)/(Fmax-F0) values against the NA concentration, where F and Fmax represent the fluorescence intensity in the presence of analyte and in the absence of analyte, respectively. Mean fluorescence responses correspond to five replicates.

Cross-reactivity determinations

The selectivity of the immunoassay was evaluated by using structurally related compounds. Stock solutions of 0.1 mg/mL of structurally related substances were prepared in ethanol. Standard curves for each of these compounds were constructed (1000–0.001 μg/L in PBS) and their inhibition concentration (IC_{50} was determined by the optimized FIA. The cross-reactivity (CR) values were calculated according to the following equation:

$$\mathrm{CR}\% = \left(NA\mathrm{IC}_{50}/\text{related compound } \mathrm{IC}_{50}\right) \times 100 \quad (1)$$

Table 2 Influence of coating antigen and antibody concentrations

Concentration of antigen (μg/mL)	Concentration of antibody (μg/mL)									
	10	20	30	40	50	60	70	80	90	100
5	4,243	4,301	4,387	4,446	4,647	4,895	4,763	4,648	4,753	4,725
10	4,357	4,402	4,242	4,363	4,751	4,923	4,800	4,804	4,972	4,989
15	4,465	4,508	4,763	4,509	4,902	5,175	5,036	5,151	5,088	4,939
20	4,536	4,676	4,790	4,948	5,271	5,364	5,221	5,062	5,090	5,111
25	4,770	4,956	4,996	4,940	5,293	5,251	5,186	5,133	5,075	5,040
30	4,847	5,047	5,147	5,150	5,305	5,313	5,153	5,197	4,994	5,132
35	4,964	5,150	5,174	5,196	5,272	5,251	5,202	5,213	4,959	4,954
40	5,114	5,259	5,219	5,241	5,311	5,298	5,204	5,198	5,186	5,227

Table 3 Influence of antigen coated time and temperature

Coat conditions	4°C				37°C			
Time(h)	8	14	24	48	1	2	4	8
$F–F_0$	4,218	4,589	4,606	4,623	4,067	4,567	4,555	4,570

Water sample analysis

Water samples were spiked with NA to evaluate potential matrix effects on the FIA. The tested waters were purified water, tap water, and river water for FIA, 10 mL of water was spiked with known concentration of each targeted compound covering the quantitative working range. In the case of river and tap water, the samples were filtered through a nylon filter. After the solution was adjusted to pH 7.0, a 0.5 mL aliquot of the sample was then mixed 1:1 with purified antiserum diluted with assay buffer, and concentration were interpolated from a PBS standard curve.

Results and discussion

Identification of the conjugates and the determination of its ratio

The reactant and product of the conjugates were scanned through UV spectra. Figure 1 shows qualitative differences between carrier proteins and conjugates in the region of maximum absorbance of hapten. Ratios of the hapten to BSA and OVA were calculated with the following formula: ratio $=\left[\varepsilon_{280\text{antigen}} - \varepsilon_{280\text{protein}}\right]/\varepsilon_{280\text{happen}}$. Respectively, their molar ratios of NA-BSA and NA-OVA were 29:1 and 14:1.

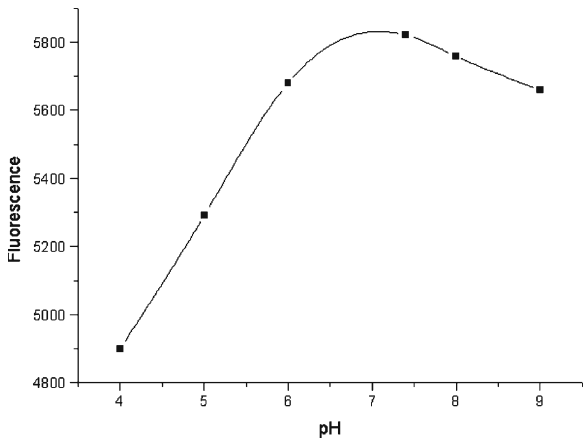


Fig. 2 Influence of pH on fluorescence

Titer of antibody

According to the agar diffusion test, when the concentration of the antigen was 0.1 mg/mL, the titers were 1:64. The FIA titers were 1.28×10^5. These data illustrated that NA-BSA conjugates is a better immunogen. Table 1 shows the results of the titers of the antiserum by agar test and indirect FIA using NA-OVA as the coating antigen.

Fluorescence immunoassay optimization

To monitor the low amounts of NA, a highly sensitive detection scheme is required, such as coating antigen concentration, dilution ratio of goat anti-rabbit IgG-FITC, length of the antigen coated, pH, and ionic strength. Concentration of Coating Antigen With goat anti-rabbit IgG-FITC dilution 1:1000, the optimal working concentrations of coating antigen and antibody were determined by checkerboard titrations (Table 2). Coated antigens were dispensed in the rows used at eight concentrations (5–40 μg/mL), and antibodies were dispensed in the columns at ten concentrations (10–100 μg/mL). The results indicate that the fluorescence intensity difference increased while the concentration of coating antigen increased and while the concentration of antibody increased. In order to get a high sensitivity and reduce the cost, the coating antigen concentration of 20.0 μg/mL and antibody of 60.0 μg/mL were recommended in this assay.

Table 4 Influence of Tween-20 on fluorescence

Concentrations of Tween-20 (*v*/*v*)	0	0.05%	0.1%	0.15%	0.2%
$F–F_0$	4354	4648	4660	4610	4639

Table 5 Influence of PBST on fluorescence

Concentrations of PBS(mol/L)	0.005	0.01	0.15	0.02
$F\text{-}F_0$	4,728	4,816	4,807	4,692

Optimization of the antigen coated

The time and temperature of the antigen coated was studied (Table 3). The results show that the antibody coated 14 h at 4°C is enough. Even it has higher fluorescence values when antigens were coated more time, but it has no significant gain of fluorescence with the time increased. It can be concluded that 14 h is long enough for the coating antigen adsorbed on wells. So the coated plate located at 4°C overnight was recommended in this assay as usual.

Effect of pH

The influence of pH was tested by preparing NA standards in pure water at different pH values in the range 4 to 9. The assay performed better in neutral or basic media, and it was inhibited below pH 6.0 (Fig. 2). The fluorescence intensity is high enough between pH 6 and 8. Once again, higher pH inhibited. It may be caused by the variously adsorbability between antigen and antibodies in different pH. For the variations of pH can affect the activities of the proteins. Therefore, a pH value of 7.4 was chosen.

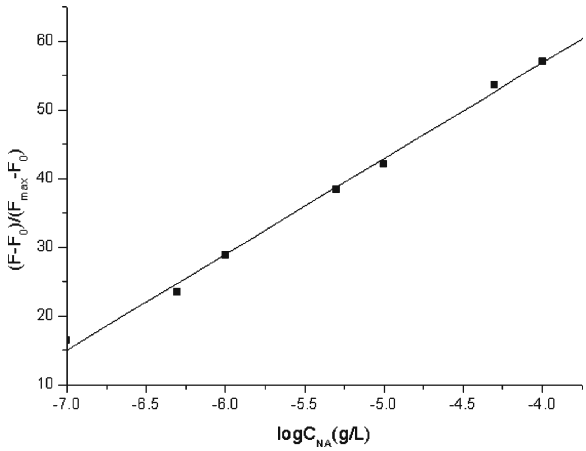


Fig. 3 Standard curve for the determination of NA by aFIA

Ionic strength

Different concentrations of Tween 20, ranging from 0 to 0.2%(v/v) were tested (Table 4). The results seem to indicate that fluorescence intensity difference increased gradually as the concentration of Tween 20 increased until the concentration reaches 0.1%. In the same way, other influence of different ionic strength were also studied (Table 5), and obtained a optimum concentration of the buffer, which provided the highest fluorescence intensity, was 0.01 mol/L phosphate buffer, pH 7.4, containing 0.1% Tween 20 for assay.

Calibration

Under the optimal conditions chosen above, the calibration graph for NA antigen was constructed (Fig. 3). The linear range was 0.1 to 100 μg/L and LOD was 0.05 μg/L. Compared with the linear range of 0.5–500 mg/L and the LOD of 8 ug/L, which have been reported by Andreolia (Andreolia et al. 1999) and Booksh (Booksh et al. 1996) separately. The method developed here has a different linear range lever and a lower LOD.

Table 6 Cross-reactivity of NA structurally related compounds

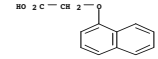
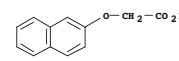
Coexisting substance	Molecule structure	Cross-reactivity(%)
Anthracene		5.7
Phenanthrene		2.6
Fluoranthene		1.8
1-naphthol		9.7
2-naphthol		11.8
1-naphthoxy acetic acid		12.5
2-naphthoxy acetic acid		14.2

Table 7 Recovery of NA from spiking water samples measured by the optimized FIA

Water samples	NA levels (μg/L)	NA Added (μg/L)	Total found (μg/L)	Recovery (%)	RSD (n = 9, %)
Double-distilled water	None detected	10	10.3	103.0	8.36
		20	21.9	109.5	6.36
		40	38.9	97.3	7.20
River water	25.6	10	40.2	113.0	4.38
		50	69.7	92.2	5.92
		100	134.1	106.8	4.95
Tap water	12.5	10	21.6	96.0	9.42
		50	63.1	101.0	10.5
		100	108.2	96.2	2.96

Immunoassay specificity

Under the optimum conditions, the potential interference of a group of seven structurally related compounds was tested. In addition, two haptens were tested. As displayed in Table 6, the antibody of this paper showed high reactivity with NA. The CRs of seven structurally related compounds were below 15%. According to the Table 6 shows that the antibody has higher CR with naphthalene derives than other PAHs.

Sample analysis

In order to assess the validity of the proposed method, NA in double-distilled water, water samples from Suzhou River (Shanghai, China), tap water were determined. Tap water and river water samples were collected in bottles, filtered and adjusted to pH 7.5 with 1 mol/L HCl or 1 mol/L NaOH, and then stored at 4°C until required.

Standards and samples were run nine times on different plates, and the mean fluorescence intensity values were recorded. The recovery rates were 92.2% to 113.0%, and relative standard deviation within a batch was below 11% (n = 9; Table 7). The results show that the recovery and reproducibility of the proposed method are satisfactory.

Conclusions

A novel fluorescence immunoassay method based on selective antigen-antibody binding has been proposed for the determination of NA. Compared with traditional analytical methods for NA such as GC and HPLC, it has the advantage of being sensitive, reliable, inexpensive, fast and simple. Some samples have been analyzed with satisfactory results to detect NA. The method has been successfully applied to determine the NA in the environmental water. New works to improve the methodology and comparisons with references methods are in progress.

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