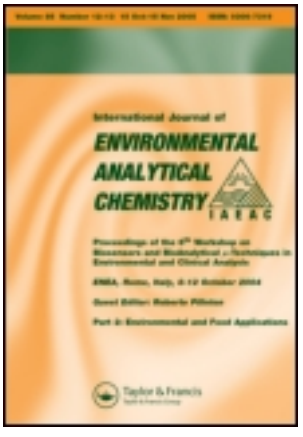

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Immunoassay for the detection of lead ions in environmental water samples

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A rapid, simple, and reliable competitive immunoassay was developed for measurement of lead ions Pb(II) in environmental samples. Avian antibodies were produced against Pb(II). Since lead ions are too small to elicit an immune response, the metal was coupled to protein carrier Bovine serum albumin (BSA) using a bifunctional chelator 1-(4-isothiocyanobenzyl) ethylenediamine *N*,*N*,*N*′, *N*′-tetra acetic acid (ITCBE). Poultry birds (layers) were immunised with this Pb(II)–ITCBE–BSA immunoconjugate and the avian antibodies (IgY) isolated from egg yolk recognised Pb(II)-ITCBE complexes as capture reagent and a Pb(II)–ITCBE conjugate of Alkaline phosphatase as an enzyme label. Antibody reaction was optimised for different concentrations of antigen and antibody dilutions. Cross reactivity with other metals were below 1% in competitive ELISA. The IC_{50} value of this avian antibody was 0.19 μg mL^{-1}. The detection range and the detection limit were 0.02–1000 μg mL^{-1}and 0.2 μg mL^{-1}, respectively.

Keywords: avian antibodies (IgY); cross reactivity; detection of lead ions; ELISA; environmental samples; specificity

1. Introduction

Lead, a heavy, soft grey metallic element, is a naturally occurring metal found in the Earth's crust in all rocks, soils and dusts. Much lead is recovered as the primary metal from galena deposits. Lead is used for weatherproofing of buildings, for equipment in the manufacture of acids and glass, as a shield against radiation in the nuclear industry, as solders in printed circuit boards and in space shuttles to encapsulate and protect the latest generation of electronic microcircuits from atmospheric corrosion [1]. Lead also plays a vital role in space exploration, energy conservation and telecommunications, development of hyper-fast computers and high-definition TV. Lead is also used in the manufacture of paints, lead acid batteries, bullets, cannon balls, lead sheet, pipes, in children's toys and as a colouring agent in ceramic glazes [2].

It has long been known that toxic effects from excessive exposure to lead can cause behavioural changes, reduction in IQ, blindness, deafness, encephalopathy, kidney failure and death [3]. Common symptoms of acute lead poisoning are loss of appetite, nausea,

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vomiting, stomach cramps, constipation, difficulty in sleeping, fatigue, moodiness, headache, joint or muscle aches, anaemia, and decreased sexual drive. Lead has been shown to have the ability to inhibit or mimic the actions of calcium (which can affect calcium-dependent or related processes) and to interact with proteins (including those with sulfhydryl, amine, phosphate, and carboxyl groups). Severe health effects of acute lead exposure include damage to the nervous system, including wrist or foot drop, tremors, and convulsions or seizures [4]. Major contributors of lead pollution are transportation sources, particularly automotive sources and industrial processes, especially metal processing, smelters, battery manufacturers and electronic wastes. When indirectly ingested through contaminated food or inhaled, lead enters the food chain from soil, water, deposition from the air, containers or dishes and from food processing equipment. Lead-contaminated food and beverages are a major source of lead exposure [3].

The introduction of improved instrumentation for the atomic absorption spectrometry and anodic stripping voltametry has made it possible to obtain accurate and precise measurement of lead. Biosensor-based detection techniques developed for the detection of Pb(II) include localised surface plasmon resonance fibre-optic Biosensor [5], DNA-based sensors [6], colloidal Au-modified piezoelectric quartz crystal (PQC) biosensor [7] and by a backward light scattering technique [8]. The presence of lead in water samples have also been reported using graphite furnace atomic absorption spectrometry after cloud point extraction [9].

Current analytical methods involving inductively coupled plasma atomic emission spectroscopy (ICPAES) [10] and graphite furnace atomic absorption spectroscopy (GFAAS) [11] for the detection of heavy metal ion residues are sensitive and reliable. They are the U.S. Environmental Protection Agency's (U.S. EPA's) methods of choice for the elemental analysis of water samples. These techniques accurately measure the level of lead in a sample; however the analysis is expensive, requires highly qualified analysts and sample preparation, and also usually requires acid digestion at elevated temperatures and pressures. In addition, the sample turnaround time is relatively short. Immunoassays offer an alternative approach, and they have significant advantages over the traditional instrument-intensive methods of metal analysis. They are remarkably quick, easily performed, reasonably portable to the contamination site, require minimum sample pre-treatment, and have high throughput. Furthermore, studies have shown that the use of immunoassays can reduce analysis costs by 50% or more [12]. Some of the immunoassays developed previously include a competitive indirect enzyme-linked immunoassay for lead ion measurement using mAbs against the lead-DTPA complex [13] and a fluorescence polarisation immunoassay (FPIA) for Lead Analysis by Anti-Chelate Fluorescence Polarisation, which has been reported by Johnson *et al.* [14]. Furthermore, most of the commercial immunoassays for environmental contaminants are directed towards halogenated or aromatic contaminants [15]. However, this technique is theoretically applicable to any pollutant, including a heavy metal, if a suitable antibody can be generated. In this report we describe the immunodetection of Pb(II) by using avian antibodies (IgY).

2. Experimental

2.1 *Materials*

All reagents were of analytical grade. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Chemical Co., MO, USA. 1-(4-Isothiocyanobenzyl)

ethylenediamine-*N*,*N*,*N*,*N*-tetraacetic acid (ITCBE) was purchased from Dojindo Laboratories (Kumamoto, Japan). Atomic spectroscopy standards, namely, lead, magnesium, manganese, zinc, cadmium, iron, nickel, and mercury (1000 μg mL^{-1} in 1 N HNO_3), were obtained from E. Merck and Inorganic Ventures. Rabbit anti-poultry IgG conjugated to alkaline phosphatase (ALP) was purchased from Bangalore Genei, Bangalore, India. ELISA microwell plates were procured from NUNC, Germany. Glass double distilled water was used in all cases unless otherwise stated. All glassware was mixed-acid washed and rinsed with purified water, and all plastic ware was soaked overnight in 3 M HCl and rinsed liberally with purified water before use. Single comb white leg horn poultry (22-week-old) were purchased from Kateel Poultry Farm, Mysore, India.

2.2 *Preparation of lead–protein conjugates*

The lead bovine serum albumin and lead alkaline phosphatase enzyme conjugates Pb(II)-EDTA-BSA and Pb(II)-ITCBE-ALP were prepared by the method [16], with a little modification. Briefly, a 53.1 mM solution of ITCBE was prepared in 0.1 M sodium phosphate buffer (pH 9.5) and the concentration was verified by measuring absorbance at 280 nm (molar extinction coefficient 17 000). Standard lead solution was dissolved in ultrapure HNO_3 and diluted to required concentration with glass double distilled water. BSA and ALP (5 mg) was mixed individually and separately in a total volume of 2 mL of a solution mixture that contained 50 mM sodium phosphate (pH 9.5), 4 mM ITCBE, and 4 mM lead. The pH of the reaction mixture was rapidly adjusted to pH 9.2 by the addition of KOH, and the solution was stirred overnight at 25°C. Unreacted ITCBE and Pb(II)-ITCBE complex were removed from the protein conjugate by buffer exchange using a Centricon-30 filter (Amicon, Inc., Beverly, MA) which had been treated with 100 mM EDTA solution and liberally rinsed with water before use. The extent of substitution of free amino groups on the BSA and ALP were determined by estimation of free amino groups on unreacted BSA and ALP and on BSA and ALP subjected to the conjugation procedure [17]. The extent of conjugation was 84.3% of the total lysine residues of BSA and ALP.

2.3 *Immunisation and antibody production*

Twenty-week-old poultry were immunised with Pb-ITCBE-BSA conjugate (1 mg mL^{-1}) in PBS and Freund's complete adjuvant. Booster doses were given once in 15 days with Pb-ITCBE-BSA conjugate in PBS and Freund's incomplete adjuvant. Injections were given intramuscularly. Eggs were collected daily and antibodies were isolated from egg yolk. Yolk was separated from egg white and suspended in PBS. The egg yolk was broken and made in to uniform suspension and homogenised well in magnetic stirrer for 10 min and was further kept for another 30 min with the addition of chloroform at ambient temperature. The mixture was centrifuged at 10,000 g, at 4°C for 10 min. The supernatant was decanted and PEG 6000 was added to the supernatant at 14% (W/V) level. This was again mixed well for 30 min at ambient temperature on a magnetic stirrer. Then it was centrifuged at 10,000 g, at 4°C for 10 min. The supernatant was decanted and the precipitate (IgY) was dissolved in required quantity of PBS and stored at −20°C.

2.4 *Optimisation of lead detection by ELISA*

The protein concentration of IgY was determined by the Bradford method [18]. The antibody concentration was determined by ELISA. Antibody (100 µl) isolated from yolk of different weeks was coated on to microwell plates and immobilised at 4°C overnight. The plates were washed with 0.1% Tween 20 in phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl), and the wells were blocked with 1% gelatin (100 µl) by incubation at 37°C for 1 h. The plates were then washed with 0.1% Tween 20 in phosphate buffered saline. Pb(II)-ITCBE-ALP conjugate (100 µl) was added to the wells of the plates and allowed to incubate in the microwells at 37°C for 1 h. After washing with PBS containing 0.1% Tween 20, pNPP in diethanolamine buffer (pH 9.8), microwell substrate (150 µl) was used for colour development. The reaction was stopped by the addition of 3M NaOH (50 µl). The absorbance of each well was measured in an ELISA reader at 405 nm. The antibody showing highest titre values were used for further studies.

The optimum antibody concentration for coating onto the microwell plates and the best working concentration of the enzyme conjugate were determined by checkerboard titration. IgY antibody was diluted with carbonate buffer (Na_2CO_3, $NaHCO_3$ pH 9.6) at concentrations from 2 µg to 1 pg (Bradford protein) and coated onto microwell plates (100 µl) by incubation at 4°C overnight. The plates were washed with 0.1% Tween 20 in phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, and 10 mM sodium phosphate, pH 7.4), and the wells were blocked with 1% gelatin by incubation at 37°C for 1 h. Pb(II)-ITCBE-ALP conjugate was serially diluted in carbonate buffer at concentrations from 2 µg to 2 pg levels (Bradford protein) and added to the wells of the plates (100 µl) and allowed to incubate in the microwells at 37°C for 1 h. The remaining procedure is as described above. Concentrations of antibody and enzyme conjugate that showed highest titre values were used for further testing.

2.5 *Immunoassay procedures*

All procedures were carried out at 37°C. Soluble atomic absorption grade lead was premixed with Pb(II)-ITCBE-ALP conjugate and aliquots (144 µg protein mL^{-1}) of the mixture were added to a microwell that had been previously coated with 1 : 50,000 dilution of IgY antibody and blocked with 1% gelatin. After 1 h incubation, the plates were washed and the amount of bound Pb(II)-ITCBE-ALP conjugate was quantified using pNPP in diethanolamine buffer (pH 9.8) microwell substrate as described above.

2.6 *Analysis of water samples*

Different types of water samples from different localities around Mysore were collected in pre cleaned polyethylene bottles with polypropylene lids and transported back to the laboratory on ice. Water was filtered through a Whatman 41 filter to remove the coarse particulates. The filtered water was then passed through a 0.45-µ syringe filter (Millipore) and stored in pre-cleaned 50 mL centrifuge tubes. A series of Pb(II)-spiked samples were prepared in the laboratory in the concentration range 0.2–200 µg $mL^{-1}$by diluting a Pb(II) standard (1000 µg mL^{-1} in 2% nitric acid) with glass double distilled water and premixing with 5 mm EDTA. A 100 µl aliquot of each sample was mixed with 100 µl of Pb(II)-ITCBE-ALP enzyme conjugate (144 µg mL^{-1}) in carbonate buffer, and 100 µl of the mixture was used for analysis by the immunoassay procedures described above.

A standard curve for Pb(II) was obtained by using Pb(II) diluted by the same procedure on plates of the same series.

Values for IC_{50} were those that gave the best fit to the following equation:

$$A = A_0 - \{(A_0 - A_1)[\text{Pb(II)}]/(IC_{50} + [\text{Pb(II)}])\}$$

where A is the signal at a definite known concentration of soluble Pb(II), A_0 is the signal in the absence of Pb(II), A_1 is the signal at a saturating concentration of Pb(II), and IC_{50} is the Pb(II) concentration that produces a 50% inhibition of the signal. The concentrations of Pb(II) in the spiked samples were then obtained by interpolation on the standard curve.

3. Results and discussion

This study describes a format for an enzyme immunoassay using avian antibodies that quantifies Pb(II) in environmental water samples. Microwell plates were coated first with avian antibody that was raised against Pb(II)-ITCBE-BSA conjugate. Distilled water containing Pb(II) was mixed with a molar excess of metal-free ITCBE to ensure that all the Pb(II) in the sample is present as an ITCBE complex, the form recognised by the antibody. This solution was subsequently mixed with Pb(II)-ITCBE-ALP conjugate, and the mixture was incubated with the immobilised antibody in the microwell. During this incubation, the Pb(II)-EDTA complexes compete with Pb(II)-ITCBE-ALP conjugate for binding sites of the immobilised antibody. After removal of unbound reagents, the amount of enzyme conjugate bound to the antibody was determined using a chromogenic substrate. The concentration of Pb(II) in a sample was quantified by the ability of its ITCBE complex to inhibit the binding of Pb(II)-ITCBE-ALP conjugate to the antibody, and colour development was inversely proportional to the concentration of Pb(II) in the original sample.

3.1 *Choice of antibody and enzyme conjugate concentrations*

The protein analysis of IgY preparations indicated that the antibody of the 14th week had the highest protein concentration. The antibody titre determination in the microwell plates indicated that the antibody of 10th-week yolks had the highest antibody titre (Figure 1). Enzyme-labelled conjugate of Pb(II)-ITCBE was prepared by reacting the isothiocyanato group of Pb(II)-ITCBE with the lysine-amino groups of the BSA and alkaline phosphatase enzyme. The conjugation reaction did not affect the enzyme activity or the immunoreactivity of the conjugate with the immobilised avian antibody.

Optimal concentrations of antibody required for coating and the best working concentration of the enzyme conjugate were determined by performing assays using varying concentrations of the conjugate and immobilised antibody. The checkerboard analysis indicated that antigen (Pb(II)-ITCBE-ALP conjugate) dilution of $1:10^3$ (1 µg protein) and antibody dilution of $1:10^5$ (144 pg protein) gave the optimum readings (data not shown). These concentrations were chosen for further work.

3.2 *Optimisation of assay condition (Calibration curves and sensitivity)*

Carbonate buffer was chosen as the buffer in the present work unless otherwise stated, 5 mM EDTA was used for subsequent experiments. Two valid calibration curves were

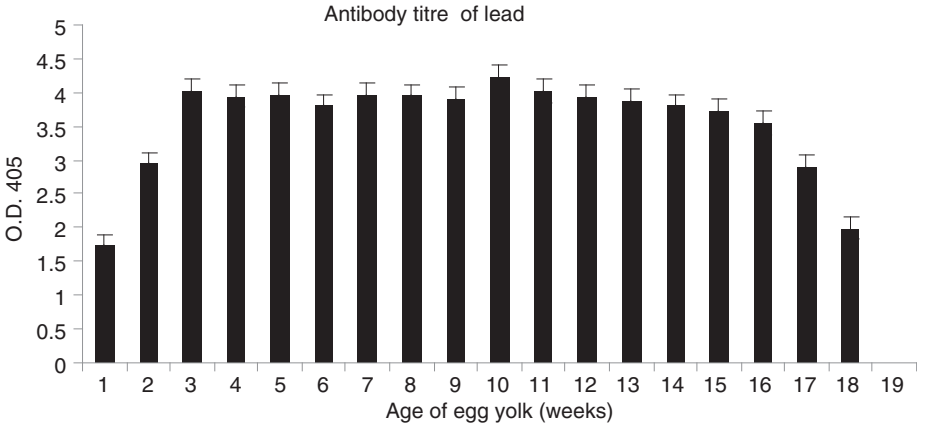

Figure 1. Antibody titre. The values are means ± SD of three independent experiments ($n = 3$).

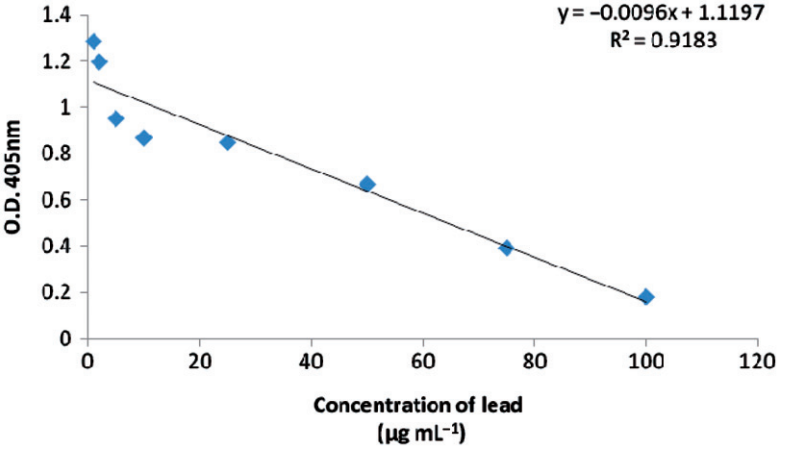

Figure 2. Calibration curve and sensitivity of the assay. The values are means ± SD of three independent experiments ($n = 3$).

generated using atomic absorption grade Pb(II) at a concentrations from 0.0 to 1000 µg mL^{-1}, prepared in distilled water and/or other water samples 5 mM EDTA (Figure 2). The sensitivity of the assay was determined by identifying the limit of detection, defined as the lowest measurable concentration of Pb(II) that could be distinguishable from zero concentration. On the basis of eight replicate measurements, the limit of detection was 0.2 µg mL^{-1}. The IC_{50} value of this avian antibody was 0.19 µg mL^{-1}.

3.3 *Metal ion specificity*

Metals are ubiquitous in the environment and interaction of the avian antibody was tested as it was necessary to test the ability of metal to cross react in the present competitive

Table 1. Metal Ion Specificity of Pb(II) Immunoassay. Competitive immunoassays were performed as described in the Experimental Section using atomic absorption grade metal ions diluted into distilled water amended with 5 mM EDTA. Values are mean of triplicate determinations.

Sl.No.	Metal	Concentration ($\mu g\,ml^{-1}$)	Cross reactivity (%)
1	Pb	2.0	100
2	Fe	2.0	0
3	Zn	2.0	0
4	Cu	2.0	0
5	Ni	2.0	0
6	Cd	2.0	0.94
7	Mn	2.0	0
8	Ca	2.0	0
9	Hg	2.0	0
10	Mg	2.0	0
11	As	2.0	0
12	Cd	5.0	2.52
13	Cd	7.5	5.51
14	Cd	10.0	12.25
15	Cd	15.0	12.31
16	Cd	20.0	12.33

immunoassay for Pb(II). Table 1 shows the cross reactivity of different individual metal ions added at 2.0 $\mu g\,mL^{-1}$ level in the assay. These data were obtained with and/or 5 mM EDTA in the assay buffer and based upon duplicate determinations. The presence of any of the metals in water did not interfere in the assay. However, Cd(II) at 2.0 $\mu g\,mL^{-1}$ level had 0.9% cross reactivity. At low concentrations of Cd(II) there was no cross reactivity. The cross reactivity increased slightly up to 10 $\mu g\,mL^{-1}$ level of Cd(II) and then remained constant (Table 1). Up to 12% cross reactivity of anti Pb(II) antibody was observed with Cd(II) metal. In a similar study the monoclonal antibody generated by fusing SP2/0-Ag14 mouse myeloma cells with spleen cells from BALB/c mouse immunised with Cd(II)-EDTA conjugated to keyhole limpet hemocyanin was checked with different metals. The presence of Ca(II), Mg(II), and Fe(III), the metal ions most commonly present at a relatively high concentrations in environmental samples, did not interfere with the Cd(II) assay over all its entire linear working range. The cross reactivity of Hg(II) was reduced from 87.64% (in assay buffer without BSA) to less than 1% when the assay buffer was supplemented with 1% BSA. The cross reactivity exhibited by any other metal ion tested was less than 3% [19].

3.4 *Precision and accuracy*

The intra- and inter-assay precisions were determined at different Pb(II) concentrations (0.2, 0.5, 1.0, 2.0, 10, and 25 $\mu g\,mL^{-1}$). The intra-assay precision was assessed by analysing eight replicates of each sample in a single run, and the inter-assay precision was assessed by analysing the same sample, as duplicates, in four separate runs. The assay gave satisfactory results over all the tested concentration levels; the coefficients of

Table 2. Precision of Immunoassay for Pb(II).

Concentration of Pb(II) ($\mu g\,ml^{-1}$)	Intraassay $n=8$		Inter assay $n=8$	
	SD ($\mu g\,ml^{-1}$)	CV[a]	SD ($\mu g\,ml^{-1}$)	CV[a]
0.2	0.07	0.35	0.06	0.3
0.5	0.11	0.22	0.21	0.42
1.0	0.21	0.21	0.49	0.39
2.0	0.58	0.29	0.86	0.43
10.0	2.55	0.25	1.22	0.12
25.0	5.41	0.21	3.88	0.11

[a]CV is the coefficient of variation.

Table 3. Comparison of Immunoassay with Atomic Absorption Spectroscopy for Analysis of water Samples Spiked with Pb(II). Competitive immunoassays were performed as described in the Experimental section. Water collected from different sources. Values are mean of duplicate determinations. All the readings were made subtracting the blank values of respective water samples.

Water sample No.	Spiked concentration ($\mu g\,ml^{-1}$)	Pb(II) ($\mu g\,ml^{-1}$)	
		Atomic absorption	Immunoassay
1	0.2	0.233 ± 0.012	0.208 ± 0.108
2	0.2	0.242 ± 0.113	0.155 ± 0.255
3	0.2	0.24 ± 0.033	0.119 ± 0.155
4	0.2	0.241 ± 0.162	0.186 ± 0.088
5	0.2	0.303 ± 0.286	0.189 ± 0.121
6	0.2	0.196 ± 0.141	0.193 ± 0.185
7	0.2	0.283 ± 0.017	0.179 ± 0.088
8	0.2	0.317 ± 0.089	0.217 ± 0.162
9	0.2	0.223 ± 0.042	0.2 ± 0.032

variations were 0.21–0.35 and 0.11–0.43 for intra- and inter-assay precision, respectively (Table 2).

3.5 *Determination of Pb(II) in environmental water samples*

Known amount ($0.2\,\mu g\,mL^{-1}$) of atomic absorption grade Pb(II) was added to water samples collected at different sources. Each sample was subsequently analysed, in duplicate, for Pb(II) content. The mean analytical recovery was calculated as the ratio, expressed as $mg\,kg^{-1}$ of Pb(II) obtained. As shown in Table 3, a quantitative recovery (98% to 158.5% and 59.5% to 108.5% respectively by AAS and immunoassay) of the added Pb(II) was obtained. Thus, the assay was able to accurately measure Pb(II) concentrations in water samples collected from different sources. The Pb(II) was spiked into different water samples having probably different concentrations of salts. Therefore the analysis by AAS and immunoassay showed differences with reference to recovery

Table 4. Comparison of immunoassay with atomic absorption spectroscopy for analysis of actual environmental water samples (without spiking). Competitive immunoassays were performed as described in the Experimental section. Water samples collected were from different localities in and around Mysore, Karnataka, India. Values are mean of duplicate determinations.

Environmental water samples (Nos.)	Quantity identified by AAS ($\mu g\,ml^{-1}$)	Quantity identified by immunoassay
1	0.088 ± 0.163	0.186 ± 0.076
2	0.096 ± 0.065	0.292 ± 0.042
3	0.143 ± 0.003	0.242 ± 0.088
4	0.149 ± 0.103	0.274 ± 0.034
5	0.236 ± 0.025	0.220 ± 0.022
6	0.272 ± 0.153	0.188 ± 0.114
7	0.311 ± 0.197	0.249 ± 0.046
8	0.037 ± 0.022	0.220 ± 0.065
9	0.192 ± 0.123	0.234 ± 0.066

of Pb(II). Boiled water (sample 8) and bore well water (sample 5) showed higher values than added $0.2\,\mu g\,mL^{-1}$ level by AAS method. But by immunoassay the interference was less in these samples (Table 3).

3.6 *Comparison of environmental water samples with atomic absorption spectroscopy*

In experiments with environmental water samples the concentration of Pb(II) was analysed by both AAS and immunoassay method (Table 4). Variation in the correlation of the values were observed. Similar variations were observed by analysis of water samples by atomic absorption spectroscopy.

4. Conclusion

A immunoassay format using avian antibodies for determination of Pb(II) in environmental water samples has been successfully developed and optimised using Pb(II)-ITCBE-ALP conjugate as an enzyme label and an immobilised avian antibody as a capture reagent. Since the assay produces a colour readout, only a colorimetric plate reader is required. The entire protocol of the present assay is very easy to perform in a 96-well plate or small strips and permits an operator to analyze a batch of 32 samples in triplicates, 48 samples in duplicate (in 96-well plate) or 8 samples, in duplicate (small strips), and obtain the results of analysis in less than 2 h when the plate has been previously coated with IgY antibody and blocked with gelatin. In addition, the assay exhibits excellent sensitivity, with the capability to determine Pb(II) in environmental water samples at concentrations as low as $0.2\,\mu g\,mL^{-1}$. This high sensitivity will enable us to validate this system for detection of Pb(II) in different water samples and different food samples. Studies are in progress to adapt this method for assessment of Pb (II) concentrations in food samples. The format is adaptable, in principle, to a wide variety of antibody-antigen systems.

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