Optimized HPLC -UV Method for Separation, Detection and Quantification of Endocrine Disrupting Estrogens in Low Quality Water

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Abstract

Endocrine disrupting estrogens are emerging contaminants in aquatic ecosystems and environment in general. There are no guidelines for routine monitoring of these chemicals, despite the existing evidences of their adverse health effect to living organisms at low concentrations. This study aimed at developing and validating an optimized HPLC-UV method for detection and quantification of estradiol and ethinylestradiol. Isocratic elution was used for separation and detection of ethinylestradiol and estradiol. The mobile phase was applied with A; water B; acetonitrile (50:50) at flow rate of 0.7mL/min and injection volume 10mL. The precision and accuracy of the method were within the acceptable range. Relative standard deviation of peak area for E2 ranged from 1.373 to 3.668%, and for EE2 ranged from 0.829 to 6.495 %. The percentage recovery for E2 ranged from 82.3 to 99.84 %, and for EE2 ranged from 84.6 to 103.52 %. Linearity of the method was realized at range of 2.5 to 50 ng/mL and 100 to 1000 ng/mL for both E2 and EE2. The linear regression coefficients were 0.9979 and 0.9973 for E2 whereas for EE2 were 0.9983 and 0.9976. Limit of detection were found to be 0.05 ng/mL and 0.08 ng/mL for E2 and EE2 respectively. The obtained limits of quantification were 0.18 and 0.28 ng/mL for E2 and EE2 respectively. In untreated sewage the concentrations of E2 and EE2 were 0.28 ng/ml and 0.18 ng/ml respectively. But in subsequent wastewater stabilization ponds the concentrations were below detection limit. Therefore, the optimized HPLC-UV method is suitable for detection and quantification of endocrine disrupting estrogens when a level of pollution is at least 0.15 ng/ml. At low extent of pollution would require use of the method in conjunction with ELISA technique.

Keywords: chromatographic method, development, validated, estrogens, analytical chemistry

1. Introduction

Estrogen hormones are emerging pollutants in water. There are natural and synthetic estrogens; the natural estrogens are not only produced by humans but also by other vertebrates as well as invertebrates particularly insects (Das, 2016). The synthetic estrogens are used for birth control, hormone replacement therapies, cancer drugs and other pharmaceuticals (Shook, 2011; Sood et al., 2014). Usually the estrogens are excreted from humans and animals mainly as conjugates of glucuronide or sulphate and a lesser amount as parent compounds in urine and faeces. The conjugates undergo deconjugation to form free estrogens by enzymatic action of bacteria or fungi (Kumar et al., 2012). The occurrence of these hormones in the environment is receiving considerable attention due to the fact that most of these compounds are endocrine disrupting chemicals (Christiansen et al., 2002; Cui et al., 2006; Zheng et al., 2013; Huang et al., 2016). Several studies have demonstrated that wastewater treatment systems are the main source of estrogens to aquatic environment (Ingrand et al., 2003; Swart & Pool, 2007; Yoon et al., 2012; Belhaj et al., 2014). A reliable analytical technique is necessary for separation, detection and quantification of the estrogens in the environmental matrix such as water.

In literature there are several analytical methods for detection and quantification of estrogens from environmental samples but the methods differ in sensitivity (Ingerslev & Halling-Sørensen, 2003). Among those methods are HPLC, LC-MS, GC-MS, and immunochemical methods. The LCMS/MS and GCMS/MS are advanced methods and most

sensitive for detection of estrogens (Faqehi et al., 2016), however their use is limited by high costs. Immunochemical methods are also sensitive but have poor selectivity compared to LCMS/MS and a single assay can only detect a single steroid (Ingerslev & Halling-Sørensen 2003; Faqehi et al., 2016).

High performance liquid chromatography is a dominant analytical technique due to its applicability to diverse analytes (Dong, 2013). The technique can quantify compounds in complex samples and can be coupled with different detectors such as UV, diode array detection (DAD), evaporation light scattering detection (ELSD), refractive index detector, fluorescence detection and mass spectrometry (MS) (Dong, 2013). The successful use of HPLC requires the right combination of variety of operating conditions such as the column length and diameter, column temperature, type of the column packing, type of the mobile phase, mobile phase flow rate, and injection volume (Settle, 2004). This implies that HPLC method development and validation are inevitable for successful application of HPLC.

Method validation ensures consistent capabilities of an analytical method by defining and confirming the analytical requirement for a specific application (Magnusson & Ornemark, 2014). The ISO/IEC 17025:2005 (2005), a guideline for competence testing and laboratory calibration, stresses that method validation is necessary requirement in analytical chemistry since it demonstrate that the method is fit for the purpose. Method validation include assessment of the parameters such as precision, linearity, accuracy, limit of detection, limit of quantification, specificity, range and robustness of the method (ICH, 2005; Magnusson & Ornemark, 2014).

Schellinger & Carr (2006) found that gradient elution gave a shorter overall analysis with similar resolution compared to isocratic elution. On the other hand isocratic elution remain preferable when the sample contains less than ten weakly retained components (Schellinger & Carr, 2006).

This study aimed at establishing an optimized HPLC-UV method for separation, detection and quantification of estrogens hormones from low quality water particularly from wastewater stabilization ponds. The study focused on the two most potent estrogens namely ethinylestradiol and estradiol.

2. Materials and Methods

2.1 Chemicals and Materials

Ethinylestradiol (EE2) and β-estradiol (E2) standards hormones were supplied by Santa Cruz Biotechnology, Texas, USA. HPLC grade methanol, acetonitrile and water (99.9% purity) were supplied by Sigma Aldrich, Germany, n-heptane (99%), acetone (99.8%) and hydrochloric acid (37%, 1.18M) were supplied by Carlo Erba Reagenti. Solid phase extraction C-18 cartridges (130 mg, 3 mL) by Varian and Chromabond, silica gel (3 ml/500 mg) from Macherey-Nagel in Germany were employed.

2.2 Preparation of Standard Solution and Calibration Standard Solution

Stock solution of 10,000 ng/ml in methanol was prepared for a mixture standard E2 and EE2, and then stored at 4°C in a fridge. Serial dilution of the stock solution was carried out to obtain 1000, 750, 500, 250, 100, 50, 25, 10, 5, 2.5, 1, 0.5, and 0.1 ng/ml for the mixture of the two hormones. In addition, separate standard solutions of the two hormones were prepared at 100 ng/mL for determination of their retention time.

2.3 Spiking Standard Estrogens in Distilled Water

For determination of recoveries the estrogens standard solutions were spiked in 1 L of distilled water to make different concentrations for both E2 and EE2. The established concentrations were 1000, 750, 500, 250, 100, 50, 25, 10, 5, 2.5, 1, 0.5, and 0.1 ng/ml. Each solution was filtered twice using by GFCs, a procedure which could be applied for removal of debris from low quality water.

2.4 HPLC Conditions

Analyses were performed at Ecotoxocology and Natural Product Laboratory at the College of Veterinary and Medical Sciences, Sokoine University of Agriculture. The HPLC (Shimadzu 20AD) fitted with an auto sampler and a SPA-UV detector at 230 nm was used for analysis. A reversed phase phenomenex C-18 column (150 x 4.6 mm and particle size of 5μm) was used for separation with the oven temperature set at 35 $^{\circ}$C. The sample injection volume was 10 μL and flow rate 0.7 mL/min. The mobile phases employed were A; water B; acetonitrile (50:50).

2.5 Method Development

With the theoretical background, detection, separation and quantification of the two estrogens hormones were achieved by manipulating factors such as wavelength, mobile phase composition, flow rate and temperature. Several trials were carried out to separate, identify and quantify a mixture of estradiol, and ethinylestradiol.

When the mobile phases were; A- water and B- methanol, the hormones were detected at shorter retention time but the resolution was very poor even after several trials upon changing column temperature, flow rate and gradient. When the

mobile phases were; A-water and B-acetonitrile, retention time increased with good resolution. It was observed that even when gradient elution was employed good resolution was obtained when equal proportions of the mobile phases run for longer time. Isocratic elution was found to be suitable over the gradient elution. Retention time for estradiol was 11.89 ± 0.03 minutes and for ethinylestradiol 14.79 ± 0.04 minutes while the method running time was 16 minutes.

2.6 Method Validation

Validation of method developed was carried out in accordance to ISO/IEC 17025:2005. Validation characteristics of the method such as precision, accuracy, linearity, specificity, limit of detection, quantification, and robustness were assessed. The procedures employed in assessment of validation characteristics were as stipulated in ICH (2005) document.

2.7 Collection of Water Samples from Wastewater Stabilization Ponds

Wastewater samples were collected from Mafisa WSPs owned by Morogoro Municipality in Tanzania. An untreated sewage wastewater samples were drawn using cleaned 2.5 liters glass bottle during dry season. Other samples were drawn from outlet of anaerobic and facultative ponds using cleaned 2.5 liters glass bottle. After sampling, the pH of the sampled wastewater samples was adjusted to about 3 by adding concentrated hydrochloric acid (1.18M) so as to fix the estrogens (Havens et al., 2010; Hansen et al., 2011). Then, the samples were carried in cool box packed with ice packs to the Ecotoxicology and Natural Products Research Laboratory in the College of Veterinary and Medical Sciences at Sokoine University of Agriculture. In the laboratory pretreatment and solid phase extraction of estrogens were conducted within 12 hours after sample collection.

2.8 Extraction of Estrogens from Water Samples

Extraction of estrogens from water samples was carried out according to the protocol described by (Hansen et al., 2011) with some modifications customized to our laboratory settings. Each water sample (1.5 litres) was first filtered twice using GFC filters papers to ensure removal of debris. The C-18 cartridges were conditioned with 2×3 mL heptane, 3 mL acetone, and lastly with 3 ml of distilled water. Thereafter, solid phase extraction (SPE) was performed with C-18 cartridges (Bond Elut 500 mg, 3cc reservoir, Varian Agilent Technologies, USA) facilitated by vacuum manifold. After extraction the cartridges were dried in air using vacuum manifold for about half an hour. Then, elution of the analyte was achieved by using 10 ml of heptane and acetone (65:35). Thereafter, the elute was air dried at 30 $^{\circ}$C, followed by reconstitution using 5 ml methanol. The samples were stored at –20 $^{\circ}$C, until analysis by HPLC-UV method.

2.9 Statistical Analysis

Microsoft excel 2007 with data analysis pack was used for statistical analysis. Descriptive statistics such as standard deviation, mean and relative standard deviation were computed.

3. Results and Discussion

3.1 Method Linearity and Range

Figure 1 and 2 demonstrate that the peak areas were directly proportional to the concentrations of E2 and EE2 respectively. Both at higher concentrations (from 100 to 1000 ng/mL) and lower concentrations (from 2.5 to 50 ng/mL) showed good linearity with linear regression coefficient being 0.9979 and 0.9973 for E2 and 0.9983 and 0.9976 for EE2. Those values of regression coefficient denote excellent linear relationship between the peak area and the concentrations of the two hormones (ICH 2005; Magnusson & Ornemark, 2014).
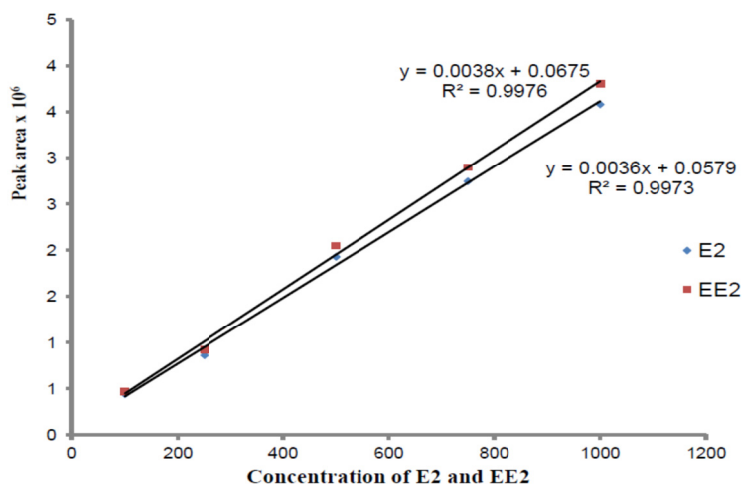

Figure 1. Calibration curves for standard E2 and EE2 at high concentrations
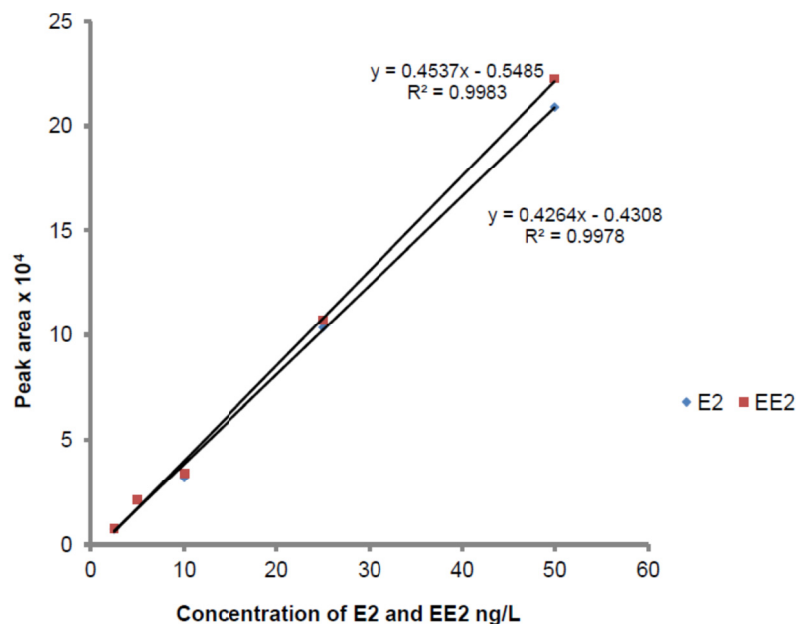
Figure 2. Calibration curves for standard E2 and EE2 at low concentrations

3.2 Method Precision

Table 1 and 2 display the method precision for detection and quantification of E2 and EE2 respectively. The relative standard deviations of peak area for both E2 and EE2 were below 2% except for the lowest concentrations which was above 2 % but below 10 %. Adebayo & Oduwaye (2015) stated that an effective HPLC method should have a relative standard deviation less than 10 % for 6 replicates. Figure 4 revealed good precision of this method when the standard solution at 100 and 250 ng/mL were analysed.

Table 1. Method Precision for detection and Quantification of E2 at Higher and Lower concentrations

S/N	(250 ng/mL		100 ng/mL		5 ng/mL		0.1 ng/mL	
	RT	PA	RT	PA	RT	PA	RT	PA
1	11.766	930,530	11.860	435,838	11.858	18,530	12.006	2,052
2	11.824	897,259	11.890	425,610	11.858	18,439	11.931	2,100
3	11.874	919,495	11.903	421,479	11.912	18,763	11.958	2,156
4	11.868	941,589	11.916	422,706	11.937	17,842	11.937	1,951
5	11.844	891,922	11.908	422,062	11.921	17,762	11.961	2,145
6	11.784	905,404	11.887	419,813	11.893	18,471	11.927	2,044
Mean	11.827	911033.17	11.894	424584.667	11.897	18,301.167	11.953	2,074.67
Std	0.044	15164.476	0.020	5830.884	0.033	403.740	0.029	76.094
RS (%)	0.374	1.665	0.167	1.373	0.278	2.206	0.246	3.668

Abbreviations: RT = retention time; PA= peak area; Std = standard deviation; RS = relative standard deviation

Table 2. Method Precision for detection and Quantification of EE2 at Higher and Lower concentrations

S/N	250 ng/mL		100 ng/mL		5 ng/mL		0.1 ng/mL	
	RT	PA	RT	PA	RT	PA	RT	PA
1	14.645	921,207	14.749	410,521	14.749	18,317	14.86	2,114
2	14.728	923,056	14.787	405,267	14.73	18,634	14.854	1,887
3	14.768	901,806	14.8	403,473	14.816	18,694	14.788	1,836
4	14.748	890,451	14.802	401,949	14.841	19,273	14.856	1,874
5	14.731	895,886	14.809	401,227	14.811	18,661	14.835	2,115
6	14.768	924,008	14.774	405,635	14.802	18,672	14.803	1,890
Mean	14.731	909,402.333	14.787	404678.667	14.792	18,708.5	14.8386	1952.667
Std	0.046	15,090.705	0.022	3352.977	0.043	310.252	0.030	126.822
RS(%)	0.310	1.659	0.151	0.829	0.289	1.658	0.201	6.495

Abbreviations: RT = retention time; PA= peak area; Std = standard deviation; RS = relative standard deviation

3.3 Method Accuracy

Table 3 and 4 display the method performance accuracy for quantification of E2 and EE2 respectively. For E2 recoveries ranged from 82.3 to 99.84 %, on the other hand recoveries for EE2 ranged from 84.6 to 103.52 %. The recoveries are within the recommended range for analytical method between 80 % to 120 % (Shabir, 2004).

Table 3. Method performance accuracy for quantification of E2 at Low & High Concentrations

Spiked Concentration ng/mL	Sample No.	Calculated amount ng/mL	% Recovery	Spiked Concentration ng/mL	Sample No.	Calculated amount ng/mL	% Recovery
50	1	49.61	99.22	1000	1	954.93	95.49
	2	49.90	99.80		2	987.00	98.70
25	1	24.74	98.96	750	1	748.83	99.84
	2	24.88	99.52		2	747.50	99.67
10	1	8.23	82.30	500	1	468.39	93.68
	2	8.97	89.70		2	480.00	96.00
5	1	4.93	98.60	250	1	239.62	95.85
	2	5.12	102.40		2	245.98	98.39
2.5	1	2.44	97.60	100	1	98.69	98.69
	2	2.49	99.60		2	99.45	99.45

Table 4. Method performance accuracy for quantification of EE2 at Low & High Concentrations

Spiked Concentration ng/mL	Sample No.	Calculated amount ng/mL	% Recovery	Spiked Concentration ng/mL	Sample No.	Calculated amount ng/mL	% Recovery
50	1	49.700	99.4	1000	1	960.46	96.046
	2	49.48	98.96		2	1002.00	100.2
25	1	24.057	96.23	750	1	753	100.4
	2	24.155	96.62		2	749.45	99.93
10	1	8.460	84.6	500	1	468.35	93.67
	2	8.860	88.6		2	485.10	97.02
5	1	5.176	103.52	250	1	240.13	96.05
	2	5.088	101.76		2	247.85	99.14
2.5	1	2.460	98.4	100	1	97.50	97.50
	2	2.478	99.12		2	99.98	99.98

3.4 Method Sensitivity

Limit of detection (LOD) and limit of quantification (LOQ) were determined using equation 1 and 2 respectively. The LOD and LOQ for E2 were found to be 0.054 ng/mL and 0.18 ng/mL respectively. The corresponding LOD and LOQ for EE2 were 0.084 ng/mL and 0.28 ng/mL respectively. This result implies that the method is suitable for moderate and higher extent of pollution of low quality water by estrogens. At low extent of pollution the method could be used in conjunction with ELISA technique. Huang & Sedlak (2001) used HPLC-UV method in conjunction with ELISA

technique for analysis of estrogens in wastewater; the results were comparable to those obtained by applying GC-MS/MS. In addition, the developed HPLC-UV method can be used in low extent pollution when standard addition technique would be applied in quantifying the estrogens. The sensitivity of this method could be comparable to HPLC-DAD method which allowed precise and accurate determination of estrogens in various environmental water at concentrations ranging between 0.025 ng/ml to 0.3 ng/ml, (Lopez and Alpha, 2001). On the other hand, this method demonstrated higher sensitivity than HPLC-UV method developed by Fonseca et al., 2013, in such method the detection limits were 0.25 ng/ml and 0.11 ng/ml for E2 and EE2 respectively. Contrary, LC-MS/MS method is more sensitive than this method, for instance Naldi *et al.*, 2016 developed LC-MS/MS method for determination of free and conjugated steroids that had LOD ranging between 0.003 ng/mL to 0.015 ng/mL for surface water and 0.014 ng/mL to 0.076 ng/mL in wastewater samples.

$$LOD = \frac{standard\ deviation}{slope} * 3 \quad \dots\dots\dots equation\ 1$$

$$LOQ = \frac{Standard\ deviation}{Slope} * 10 \dots equation\ 2$$

3.5 Robustness

The method gave comparable results at different wavelengths such as at 215, 230, and 254 nm. Likewise when column temperature was varied from 35° C to 40° C no significant change of result occurred. Contrary to that retention time changed with change in flow rate for instance at flow rate 1 mL/min retention time was found to be 8.3 and 10.4 minutes for E2 and EE2 respectively. Whereas at a flow rate of 0.7 mL/min the retention time is 11.89 ± 0.03 and 14.79 ± 0.04 minutes for E2 and EE2 respectively.
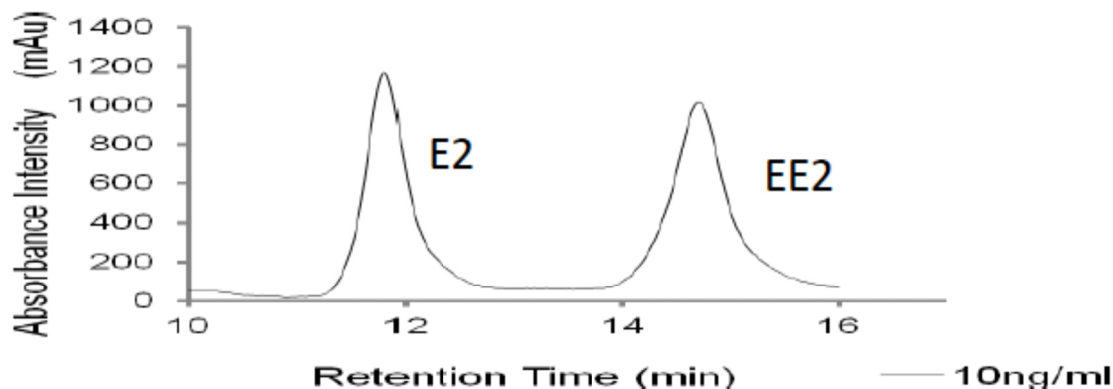

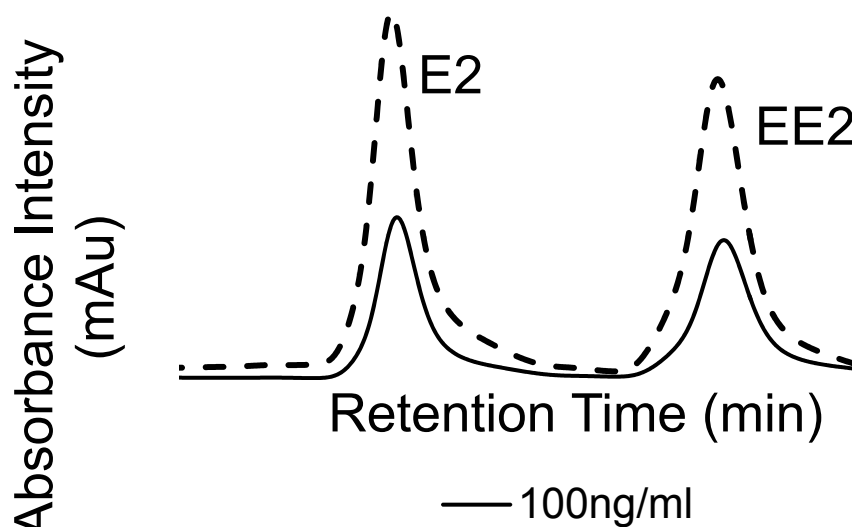Figure 3. Chromatogram of E2 and EE2 when concentration was 10 ng/mL

Figure 4. Chromatogram of E2 and EE2 at 100 ng/ml and 250 ng/ml

3.6 Levels of Estrogens in LQW Detected through HPLC-UV Method

Estrogens were detected in untreated sewage sample but in the outlets of anaerobic and facultative ponds were below the detection limit. The concentration of E2 and EE2 in untreated sewage was found to be 0.28 ng/ml and 0.18 ng/ml respectively. When ELISA competitive technique was employed revealed that the levels of estrogens in the study area were below the detection limit of the developed method. The concentrations EE2 and E2 at Mafisa WSPs ranged from 0.036 ng/ml to 0.0015 ng/ml and 0.086 ng/ml to 0.0044 ng/ml respectively (Msigala et al., 2017).

4. Conclusion

This paper presents a fast, linear, precise, accurate and robust HPLC-UV method for separation, detection and quantification of estrogens hormones from low quality water. The method can give precise and accurate results for moderate and higher extent of pollution of low quality water by estrogens. At lower extent of pollution (below 0.15 ng/mL) can be used in conjunction with ELISA technique, in such combination the HPLC method offset the shortcomings of the results which could be obtained through ELISA alone, on the other hand utilize the high sensitivity of ELISA technique.

Conflict of Interest

The authors declare that there are no conflicts of interest which could potentially influence this work

Acknowledgments

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