

Determination of Estrogens in Raw and Treated Wastewater by High-Performance Liquid Chromatography-Ultraviolet Detection

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Abstract

Determination of natural and synthetic estrogens in Waste Water Treatment Plants (WWTP) is fundamental for risk assessment regarding the endocrine disrupting effects in the aquatic environment. A methodology of analysis based on HPLC procedure with UV detection and a C18 analytical column has been developed for the simultaneous determination of estrogens in water samples in less than 9 minutes. The estrogens analysed were estrone, 17 β -estradiol, 17 α -ethinylestradiol and estriol. A pre-concentration of the analytes in water samples, was achieved using a SPE procedure with polymeric Strata-X cartridges. Average recoveries of the hormones ranged between 85-120% of concentrations tested. The use of SPE-HPLC method allows limits of detection of 0.089 $\mu\text{g L}^{-1}$ for estrone, 0.25 $\mu\text{g L}^{-1}$ for estradiol, 0.45 $\mu\text{g L}^{-1}$ for estriol and 0.11 $\mu\text{g L}^{-1}$ for ethinylestradiol. Samples collected from nine WWTP's raw wastewaters and treated wastewaters located at the Portugal Central Region, were analyzed and the concentrations obtained were in the range of 0.15-0.72 $\mu\text{g L}^{-1}$ for estrone, 0.10-0.51 $\mu\text{g L}^{-1}$ for estradiol, and 0.11-0.23 $\mu\text{g L}^{-1}$ for ethinylestradiol.

Keywords: Estrogens; SPE; HPLC-UV; Water analysis; WWTP's raw wastewaters; WWTP's treated wastewaters

Introduction

During the past few years, there has been a growing concern on possible harmful consequences of exposure to estrogens which are capable of modulating or disrupting the endocrine system [1].

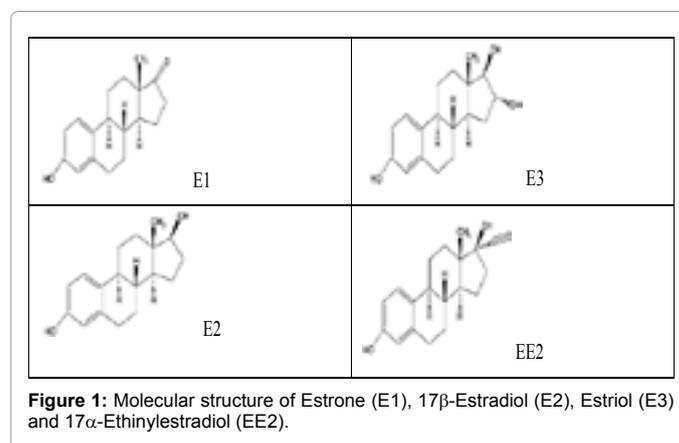
Endocrine disrupting chemicals are of increasing concern due to the risk to human life and to wildlife health. These chemicals include a wide range of compounds such as natural and synthetic estrogens, pharmaceuticals and pesticides. These molecules can disturb the normal activity of the endocrine system, and they have been continuously discharged into the aquatic environment without restrictions. The presence of these compounds in water can cause several adverse effects in the physiology of humans or other organisms in the waters, such as fish [2]. So, recently much research has been done in direct consequence of the occurrence, effects and risks of these compounds. The occurrence and destiny of these compounds in the environment has become a subject of public interest and research [3,4]. Estrogens monitoring in the environment has become of great interest, mainly due to frequent detection in treated wastewaters of Waste Water Treatment Plants (WWTP's), waters for human consumption, rivers and lakes, at concentrations in the range of ng L^{-1} to $\mu\text{g L}^{-1}$. Estrone (E1), 17 β -estradiol (E2) and estriol (E3) are natural female sex hormones produced by humans, mammals and other vertebrates. Ethinylestradiol (EE2) is a synthetic estrogen that has therapeutic uses, such as oral contraceptive (Figure 1) [1,4].

These natural and synthetic estrogens are known to contribute to a large extent of the estrogenicity of WWTP's treated wastewaters; many of their constituents are excreted unchanged as well as metabolites [5,6]. WWTP's are large non-linear systems subject to large perturbations in flow and weight, together with uncertainties concerning the composition of the raw wastewater [7-9]. Inactive hormones could be converted back in the environment to their active forms, because they not completely degraded biologically.

The estrogens have been detected in waste waters, surface waters as well as in groundwater, and are considered the most important

compounds responsible for the in vitro estrogenic activity of treated wastewaters of domestic WWTP's [9,10]. The decrease of fertilization rate and the alteration of reproductive performance of fishes, as well as various development and reproductive capacities of other aquatic invertebrates have been also reported [8,10]. The behaviour and fate of these estrogens depend on its physical-chemical properties and on the environment characteristics [5,10,11].

In order to prevent these uncontrolled effects on human health and



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the deleterious effects on the aquatic environment, great importance should be attached to the careful monitoring required for risk assessment and remediation [12,13].

In the last few decades, many efforts have been devoted to the development of analytical methodologies for detection of the estrogenic compounds in environmental samples. Accordingly, there is considerable interest in developing sensitive analytical methods applicable to estrogens monitoring. Most methods of determination of emergent contaminants reported in the literature use HPLC-MS, however this technique is expensive comparing to HPLV-UV. HPLC is enabling to determinate estrogenic compounds with limits of detection between 0.3 and 1.1 $\mu\text{g L}^{-1}$ using UV detection [14,15]. Since these pollutants are usually present in aqueous samples at low concentrations levels, a simple extraction and pre-concentration step is needed prior to measurement [6,16].

Solid phase extraction (SPE) is recognized as a very common sample pre-treatment methodology for concentrating the target analytes in biological and environmental samples. SPE coupled with HPLC is an interesting method for determination of trace organic compounds, and combines the advantages of SPE and those of HPLC.

The first aim of this study is to optimize a SPE procedure for cleaning and concentrating samples together with an HPLC methodology for the simultaneous determination of these four estrogens in wastewater samples. The second aim was quantify these estrogens, on nine WWTP's, located at the Portugal Central Region, before discharge into the Rivers.

Experimental

Instrumentation

A high-performance liquid chromatographic (HPLC) (Dionex) system equipped with four P680 pumps, an automatic injector ASI-100, a loop of 100 μL and a PDA-100 ultraviolet detector was used. All separations were achieved on an analytical reversed phase C18 column (150 mm x 4.6 mm x 5 μm , Restek Pinnacle, DB) with a mobile phase flow rate of 0.8 mL min^{-1} under isocratic conditions. The mobile phase was a mixture of acetonitrile/water (45:55; v/v). The amount of sample injected was 20 μL and the detection wavelength used was 220 nm.

For SPE Visiprep larger volume sampler and visiprep SPE vacuum manifold from Sigma-Aldrich (NSW, Australia) were used.

The Strata-X 33 μm Polymeric Reversed Phase Column (500mg/6mL) was obtained from Phenomenex (USA).

Chemicals and reagents

A stock standard solution (0.25 g L^{-1}) of Estrone (Sigma, 99%), 17 β -estradiol (Fluka, 97%), estriol (Sigma, 99%) and 17 α -ethinylestradiol (Sigma, 98%) was prepared in acetonitrile (Panreac Química, S.A.). From stock solution, standards with concentrations in the range 0.50 to 10.00 mg L^{-1} were prepared in water. The solutions were degassed in an ultrasonic bath for 15 min before use, and filtered through a 0.22 μm membrane filter prior to analysis in HPLC.

Preparation of WWTP's samples

Water samples, with a volume of 1000 mL were collected from the entrance and treated wastewater of nine main WWTP's, three times each and three replicates each), located at the Portugal Central Region, receiving only domestic waters. All WWTP'S studied have secondary treatment: primary treatment is intended to reduce oils, grease, fats,

sand, grit, and settle-able solids (this step is done entirely mechanically by means of filtration and sedimentation) and secondary treatment planned to substantially degrade the organic content of the sewage. The organic solids (sludge) are neutralized and then disposed or re-used. The final treated wastewater is discharged into the Rivers. However, WWTP's are non-linear systems subject to great perturbations in flow and weight, together with uncertainties concerning the composition of the raw wastewater.

All water samples were filtered through glass microfiber filter GF/F 55 MM from Whatman with vacuum pump, to eliminate suspended solids, and passed successively through a glass fiber 0.45 μm from Millipore, before SPE procedure. Samples were acidified to pH 2 and stored at 4° C in the dark. The SPE procedure used for the samples was always carried out within 24 h after collection to keep microbial degradation to a minimum. After SPE procedure, the eluate was dried and the extract redissolved in 1 mL of acetonitrile and filtered through a 0.22 μm membrane, from Millipore, filter prior to analysis by HPLC.

HPLC-UV analysis

To optimize the HPLC conditions in order to determine the efficiency of the method, standards of mixtures of the estrogens was analysed. The elution conditions used to separate the compounds gave a good resolution (Figure 2).

The limits of detection (LOD) for each estrogen were determined according with the equations bellow [16]

$$y = a + b x$$

$$\text{LOD} = Y_B + 3S_B \quad [\text{Eq 1}]$$

$$Y_B = a;$$

$$S_B = S_{y/x}$$

Where:

LOD - value of y at the limit of detection;

$S_{y/x}$ - Standard Deviation in the y-direction

The instrumental LOD obtained were 0.45 mg L^{-1} for E3, 0.24 mg L^{-1} for E2, 0.11 mg L^{-1} for EE2 and 0.089 mg L^{-1} for E1. The correlation coefficients of the calibration lines were higher than 0.999.

Optimization of solid-phase extraction procedure

Five methods of SPE were tested (Table 1). Different types of conditioning and washing of cartridges were tested as well as the pH of standard solution and the type of solvent to elute. In the five tested

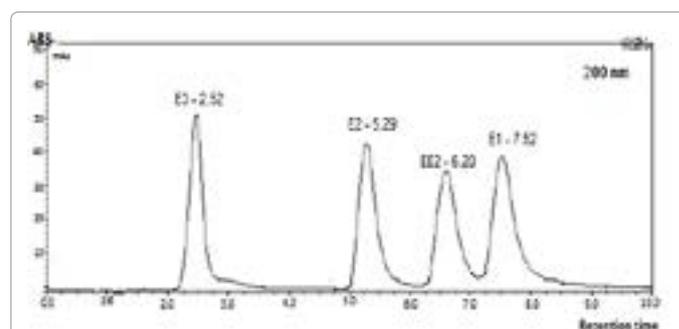


Figure 2: Chromatogram of a standard of the estrogens at concentrations of 10 mg L^{-1} .

methods, a 1000.0 ± 0.4 mL solution standards of estrogens with concentrations of 20 µg L⁻¹ each, acidified with hydrochloric acid 0.1 mol L⁻¹, were passed through the cartridges at a flow rate of 4 ml/min using a pressure of 400 mbar. The choice of pH value of the solutions used for each method tested was based on references [5,7,16-19]. After elution, samples were dried using vacuum, and then redissolved in 2 mL of methanol or acetonitrile before analysis.

Results and Discussion

Recovery of estrogens using the SPE procedure

SPE recoveries of the four estrogens were calculated by comparing the amounts of estrogens in standards before and after being subjected to SPE procedure (Table 2).

The method 2, at pH 2, was chosen since it showed highest recovery rates, ranging from 100 to 120% for E1, 85 to 110% for E2, 90 to 120% for EE2 and 89.8 to 110% for E3. In this method the cartridges were conditioned with 5 mL of methanol, followed by 5 mL of pure water. A sample of 1000.0 ± 0.4 mL, acidified at pH 2 with hydrochloric acid 0.1 mol L⁻¹, was passed through a SPE cartridge with a flow rate of 4 mL/min and a pressure of 400 mbar. Afterwards, 5 mL of water was used as a cartridge cleanup. The cartridges were dried using vacuum for 2 hours and the elution performed only on the following day.

The elution was performed with 10 mL of acetonitrile. The eluate was dried completely under vacuum and the extract redissolved in 2 mL of acetonitrile, filtered through 0.22 µm membrane filter and analysed by HPLC.

For recoveries test, distilled water (1000.0 ± 0.4 mL) was spiked with

the target estrogens in order to obtain, before SPE procedure, initial concentrations of 20 µg L⁻¹, 10 µg L⁻¹ and, 1 µg L⁻¹ (Table 3). For each concentration, the procedure was repeated three times and analysed by HPLC-UV three times.

Recoveries for the four estrogens were shown to be satisfactory (85% -120%) in the range of concentrations tested. The RSD of all recovery experiments was less than 1.47%, with a large replicate of samples (90%) with RSD < 0.79%. Correlation coefficients were obtained by plotting the area of the analyte peak versus spiked concentrations obtained (after SPE procedure). The values of correlation coefficients for each estrogen were higher than 0.998, considered very good.

Analysis of samples from raw wastewater and treated wastewater of WWTP's

The method SPE-HPLC was applied for the determination of the selected estrogens in nine WWTP's receives domestic waters and discharge treated wastewaters. Results are reported in Table 4. General raw wastewater's estrogens concentrations are in the ranged from, 0.14 to 0.51 µg L⁻¹ for estradiol (with a retention time ± 5.29), 0.13 to 0.23 µg L⁻¹ for ethinylestradiol (with a retention time ± 6.20) and 0.16 to 0.72 µg L⁻¹ for estrone (with a retention time ± 7.52). Treated wastewater's estrogen concentrations are in the ranged from 0.10 to 0.20 µg L⁻¹ for estradiol (with a retention time ± 5.29), 0.11 to 0.19 µg L⁻¹ for ethinylestradiol (with a retention time ± 6.20) and 0.15 to 0.25 µg L⁻¹ for estrone (with a retention time ± 7.52).

Although the E3 presented a peak in the chromatograms, the small area obtained resulted in a value below LOD of respective calibration curve. In order to confirm if each peak area corresponding only to each

	Method 1	Method 2	Method 3	Method 4	Method 5
Conditioning of cartridges	2×4 mL methanol 2×4 mL water	5 mL methanol 5 mL water	2×2.5 mL acetone 2×2.5 mL methanol 2×5 mL water	3×2 mL hexane 2 mL acetone 3×2 mL methanol	3 mL methanol 3 mL water
pH of estrogens standard solutions	3	2	5	3	2
Washing step	2×5 mL methanol:water (1:1) 2×5 mL acetone:water (1:2)	5 mL water	25 mL hexane and water (1:4)	5×2 mL water	2×10 mL water
Elution	2×4.5 mL methanol	10 mL aceto- nitrile	2×10 mL acetone	4 x1 mL acetone	20 mL methanol
Complete dryness of solvents	Vacuum	Vacuum	Vacuum	Vacuum	Vacuum
Redissolution of estrogens	2 mL methanol	2 mL acetonitrile	2 mL methanol	2 mL methanol	2 mL methanol

Table 1: SPE methods for concentrating estrogens using standards of 20.00 µg L⁻¹ of each compound.

Recoveries %	Method 1	Method 2	Method 3	Method 4	Method 5
Estrone (E1)	25-35	100-120	60-70	40-50	120-140
RSD %	23.5	12.8	10.8	15.7	10.8
Estradiol (E2)	26.1-33	85-110	61-68	41.5-49.2	110-135
RSD %	16.5	18.1	7.6	17.0	14.3
Estriol (E3)	25.2-29	89.8-110	65-70	42-49	111-135
RSD %	9.9	14.2	5.2	10.8	13.7
17α-ethinylestradiol (EE2)	27-34.5	90-120	64.2-70	45-49	121-138
RSD %	17.2	18.2	6.1	6.0	9.28

Table 2: Recoveries of SPE procedure for target estrogens using standards with a concentration of 20.00 µg L⁻¹ (n=3).

Matrix	Concentrations tested	Estrone		Estradiol		17α-ethinylestradiol		Estriol	
		Recovery %	RSD (%)	Recovery %	RSD (%)	Recovery %	RSD (%)	Recovery %	RSD (%)
Distilled Water sample	20 µg L ⁻¹	110	0.3	115	0.42	116	0.65	101	1.47
	10 µg L ⁻¹	110	0.06	111	0.79	120	0.72	97	0.05
	1 µg L ⁻¹	115	1.02	106	0.06	110	1.32	85	0.10
Correlation coefficients		0.9977		0.9993		0.9993		0.9998	

Table 3: Recovery data for target estrogens in distilled water after SPE at different initial concentrations (n=3).

estrogen detected, samples were spiked with a known concentration ($1 \mu\text{g L}^{-1}$) of the four target estrogens. All the samples were reanalysed to evaluate the increase of peaks area, and calculated the difference between the first and second area (sample and spiked sample).

The estrogen more frequently detected in raw wastewater's of all WWTP's were EE2 and E3 (bellow LOD), but in treated wastewater's the highest concentration was E1 (Figure 3). As expected natural estrogens and synthetic contraceptive estrogen, were identified in WWTP's raw wastewaters who serve more people. Their presence may

be explained because tested raw wastewaters contain urban discharge where these estrogens are certainly present as consequence of human excretion.

The presence of EE2 in treated wastewaters is of particular interest as it exhibits toxicity at low nanogram per liter levels [20]. E1 and E2 are the most abundant estrogen founded in treated wastewater's, but only in plants serving more people.

So, the occurrence of E1, E2 and EE2 on treated wastewater's plants may be the result of incomplete removal of these compounds during

Estrogens concentrations	WWTP 1		WWTP 8		WWTP 9		WWTP 3		WWTP 6		WWTP 7		WWTP 5		WWTP 2		WWTP 4	
	Jan-Mar		Jan-Mar		Jun-Aug		Feb-Apr		Feb-Apr		Jun-Aug		Apr-Jun		Oct-Dec		Apr-Jun	
	Number of people served by each WWTP																	
	3 000		3 000		3145		5 000		6 000		20 500		50 000		180 000		203 202	
	IIn	IOut	OOut	IIn	IIn	OOut	IIn	OOut	IIn	OOut	IIn	OOut	IIn	OOut	IIn	OOut	IIn	OOut
E3 ($\mu\text{g L}^{-1}$)	0.24	0.15	0	0.15	0	0	0.25	0	0.16	0	0.15	0	0.14	0	0.24	0.12	0	0
SD	± 0.01	± 0.02	0	± 0.02	0	0	± 0.03	0	± 0.04	0	± 0.02	0	± 0.01	0	± 0.02	± 0.02	0	0
E2 ($\mu\text{g L}^{-1}$)	0	0	0	0	0	0	0	0	0.25	0.19	0	0	0	0	0	0	0.14	0.1
SD	0	0	0	0	0	0	0	0	± 0.02	± 0.03	0	0	0	0	0	0	± 0.03	± 0.01
EE2 ($\mu\text{g L}^{-1}$)	0.13	0.14	0	0.14	0	0	0.16	0	0.19	0	0.14	0	0.18	0	0.15	0.12	0.14	0
SD	± 0.01	± 0.03	0	± 0.03	0	0	± 0.02	0	± 0.02	0	± 0.03	0	± 0.02	0	± 0.02	± 0.02	± 0.06	0
E1 ($\mu\text{g L}^{-1}$)	0	0	0	0	0	0	0.23	0.16	0.16	0.15	0	0	0	0	0	0	0.72	0.25
SD	0	0	0	0	0	0	± 0.02	± 0.01	± 0.04	± 0.02	0	0	0	0	0	0	± 0.12	± 0.02

Table 4: Occurrence of selected estrogens concentrations, expressed in $\mu\text{g per liter}$ ($n=3$), in raw wastewater's WWTP's raw wastewater's and treated wastewater's of nine plants investigated.

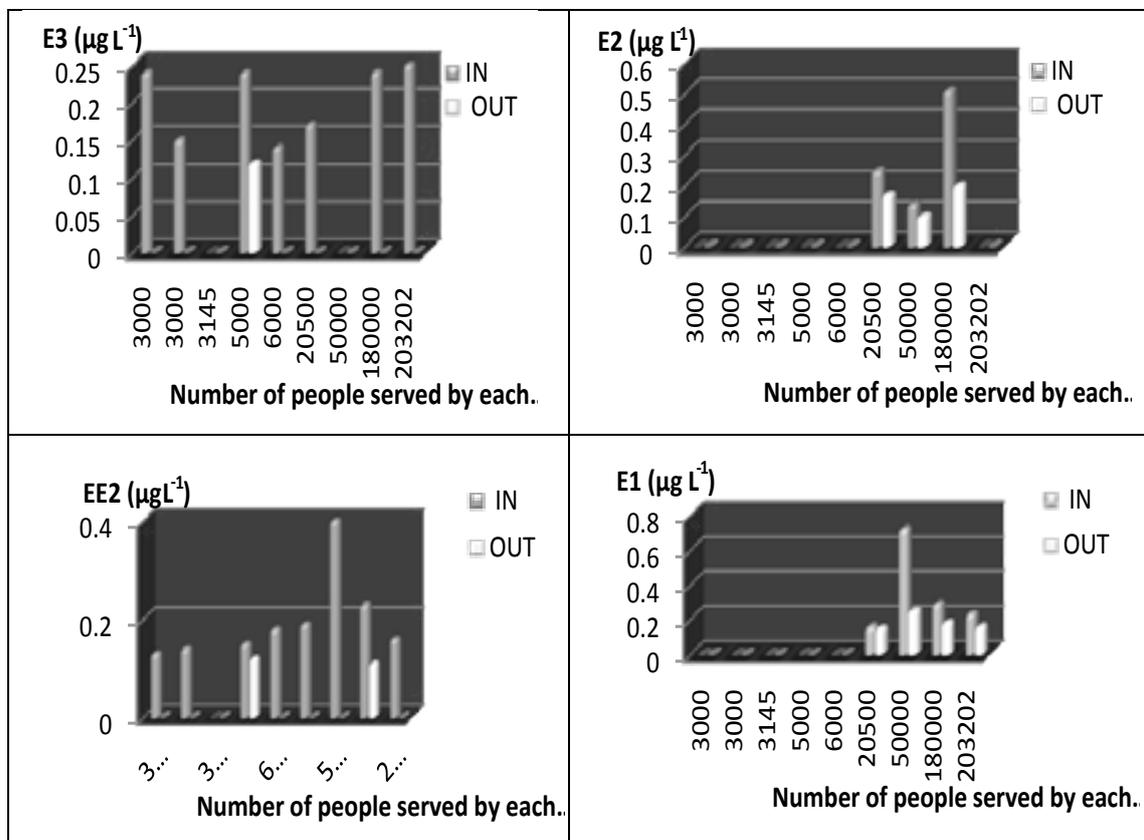


Figure 3: Graphics of each estrogen vs. raw wastewater and treated wastewater of nine different WWTP's.

treatment or release of the active estrogenic forms from conjugates during the same treatment process [20,21].

Conclusions

Most current WWTP's are not really designed to treat organic contaminants and a high part of emerging compounds and their metabolites may escape elimination in WWTP's and enter the aquatic environment via sewage treated wastewaters. The natural estrogens as well as the contraceptive estrogen were frequently detected in WWTP's discharges, due to their incomplete removal during the treatment of waste water [6,16-19].

In this study, an optimization of the analytical method based on a SPE-HPLC procedure with UV detection has been developed for the simultaneous determination of estrogens in waste water samples.

Five different SPE methods were tested, with standards, for the optimization of SPE procedure. For the chosen method, standards were used for recovery evaluation, with results ranging 100 to 120% for E1, 85 to 110% for E2, 90 to 120% for EE2 and 89.8 to 110% for E3.

These results confirm that SPE-HPLC method is therefore considered to be suitable for the application in environmental water samples. The optimized method was used for the determination of all targeted estrogens in all raw wastewater's and treated wastewater's wastes water samples.

All the compounds that were selected in the study were found in raw wastewaters and treated wastewaters in concentrations varying in the microgram per liter range, indicating a permanent enter in rivers, where these treated waters are always discharged.

General raw wastewater's estrogens concentrations are in the ranged from 0.14 to 0.51 $\mu\text{g L}^{-1}$ for estradiol, 0.13 to 0.23 $\mu\text{g L}^{-1}$ for ethinylestradiol and 0.16 to 0.72 $\mu\text{g L}^{-1}$ for estrone. Treated wastewater's estrogens concentrations are in the ranged from 0.10 to 0.20 $\mu\text{g L}^{-1}$ for estradiol, 0.11 to 0.19 $\mu\text{g L}^{-1}$ for ethinylestradiol and 0.15 to 0.25 $\mu\text{g L}^{-1}$ for estrone. These results are similar when compared to values obtained in other countries, such as Germany, Italy and Netherlands [11,22-24].

The results show that the studied estrogens can be simultaneous separated and determined from waste water samples by the proposed method with satisfactory accuracy and precision. We think it should be a systematic monitoring in the determination of estrogens in all WWTP's, who serving people, in a public health issue and as a future work.

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