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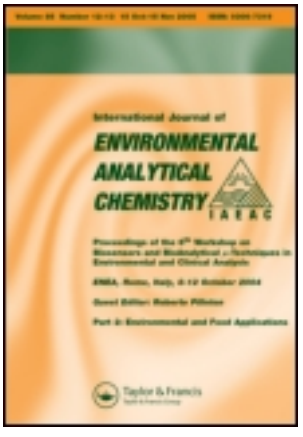

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The application of hollow fibre-liquid phase micro-extraction on the bioassay experiment of oestrogen chemicals

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The application of hollow fibre-liquid phase micro-extraction on the bioassay experiment of oestrogen chemicals

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The object of this study was to improve pre-concentration techniques using hollow fibre protected-liquid phase micro-extraction (LPME) for its application on biotic samples and limited environmental samples provided in small volumes. Five parameters, including extraction time, type of solvent, extents of agitation, fibre length and salt content of HF-LPME were optimised for the yeast two-hybrid assay and gas chromatography-mass spectrometer (GC/MS) chemical analysis. The results of chemical analysis were compared to the results of a bioassay using the yeast two-hybrid system to analyse endocrine-disrupting chemicals (EDCs). For the yeast two-hybrid assay, the optimised parameters comprised of an extraction time of 40 min, toluene as the extraction solvent, a stirring rate of 500 rpm, a fibre length of 2.0 cm and a salt concentration of 20% (w/v). For GC/MS analysis, HF-LPME conditions were optimised with an extraction time of 40 min, toluene: dichloromethane at a ratio of 1 : 1 (v : v) as the extraction solvent, a stirring rate of 700 rpm, a fibre length of 2.0 cm and a salt concentration of 30% (w/v). The results indicated that fibre length, extraction time, and degree of agitation were major contributing parameters for HF-LPME in EDCs analysis. Consequently, bioassays such as yeast two-hybrid assay could be easily, rapidly, and inexpensively conducted with small volume of sample using HF-LPME for the measurement of oestrogenic activity.

Keywords: hollow fibre-liquid phase micro-extraction; endocrine disrupting chemicals; oestrogenic activity; yeast two-hybrid assay

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1. Introduction

A number of pre-concentration techniques have been developed for the effective detection of various chemicals in aqueous samples. Of these, liquid-liquid extraction (LLE) and solid phase extraction (SPE) have been conventionally and widely used for chemical analyses regardless of the advanced development of analytical instruments. However, these techniques are known to have drawbacks such as being time-consuming, labour-intensive, and requiring a high volume of hazardous organic solvents. The use of harmful compounds and large amount of solvents can be additional environment pollutants. As a means of overcoming these problems, miniaturised extraction techniques have been developed such as solid-phase micro-extraction (SPME) [1,2] and liquid phase

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micro-extraction (single-drop LPME) [3]. In case of LPME technologies, a number of subcategorised techniques such as single drop micro-extraction (SDME), continuous flow micro-extraction (CFME), directly suspended droplet micro-extraction (DSDME), and hollow fibre-liquid phase micro-extraction (HF-LPME) have been developed for better methodological validation [4–6]. These miniaturised techniques are more convenient than the conventional methods though they also have inherent limitations. The extract quality of SPME is dependent on the fibre quality, and the fibre can be easily damaged by soil particles in the sample [7,8]. In single-drop LPME, it is hard to mechanically stabilise organic solvents at the tip of a syringe. Thus, the organic solvent can be easily lost in the sample during extraction [4].

Hollow fibre-protected LPME was introduced by Pedersen-Bjergaard and Rasmussen as a means of overcoming some of the limitations of conventional techniques [9]. In addition, an impregnated hollow fibre creates a thin liquid membrane between the aqueous sample and the acceptor solvent, which then protects the acceptor solvent from the agitated aqueous sample [4–6,10]. Since only a small volume of both the sample and organic solvent are needed for HF-LPME, this method can be effectively used in both environmental samples and biotic samples such as human plasma, whole blood, urine, breast milk and saliva [11–15]. Moreover, application of HF-LPME on detecting various target compounds including non-steroidal anti-inflammatory drugs, antibiotics and heavy metals has been recently tried [12,15,16].

In recent years, many studies have been focused on the adverse effects of endocrine-disrupting chemicals (EDCs) on the aquatic ecosystem due to their high oestrogenic activities [17,18]. A chemical analysis system for monitoring endocrine disruptors in aqueous and biological samples was subsequently developed [19–21]. The chemical analysis monitors the exact concentration of oestrogenic compounds in the samples. However, there is a limitation of chemical analysis unaccompanied with bioassay. That is, the biological effect of individual oestrogen, mixture effect and the contribution of individual compounds to overall oestrogenicity are not predicted exactly by chemical analysis alone. The chemical analysis does not guarantee the biological effects generated within an *in vitro* system in the real environment because of the existence of a number of unknown chemicals. Thus, there is a certain degree of uncertainty in the monitoring of synergistic and anti-oestrogenic effects by chemical analysis alone in a mixture of oestrogenic compounds. There is a need, therefore, for bioassays to be integrated into monitoring of oestrogenicity. Several types of bioassays have been developed for measuring oestrogenic activity, generally based on the mechanism of activation of oestrogen receptor [22–24]. The yeast two-hybrid assay, an *in vitro* technique, was chosen for this study since it can be accomplished with a very small volume of media and sample and within a short period of time. Furthermore, since the co-activators and oestrogenic receptors are recombined in a yeast plasmid, it bears more similarity to the mammalian hormonal systems than other existing bioassays using yeast [25]. In order to obtain small volume of samples extracted efficiently, a new pre-treatment method (e.g. HF-LPME) for bioassay was needed in comparison with SPE or LLE.

The objectives of this study were to determine the applicability of HF-LPME pre-treatment techniques on the analysis of oestrogenic chemicals by chemical and biological methods. For the purpose of this study, five parameters of HF-LPME were optimised for the GC/MS chemical analysis and yeast two-hybrid assay. Then, the oestrogenicity of target EDCs through the HF-LPME was compared to solid phase extraction (SPE).

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2. Experimental

2.1 *Chemicals and reagents*

E2 (17β-estradiol, CAS: 50-28-2; $\geq$ 98% purity), EE2 (17α-ethinylestradiol, CAS: 57-63-6; $\geq$98% purity) and E1 (Estrone, CAS: 53-16-7; $\geq$98% purity) were purchased from Sigma (St. Louis, MO, USA), and their structural and physic-chemical properties were described in Table 1 [23,26–27]. Pyrene-d_{10} (CAS: 490695) and bisphenol A-d_{16} (BPA-d_{16}; CAS: 451835) (purchased from Aldrich (Isotec), Miamisburg, OH, USA) were used as surrogate and standard chemicals, respectively. Dimethylsulfoxide (DMSO) for yeast two-hybrid assay was purchased from Sigma (St. Louis, MO, USA), dichloromethane from Fisher Scientific (Pittsburgh, PA, USA), and toluene from Aldrich (Milwaukee, WI, USA).

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2.2 *Sample preparation by HF-LPME*

The experimental set-up of HF-LPME and its basic principle is illustrated in Figure 1. A 10-µl microsyringe was purchased from Agilent Technologies (Palo Alto, CA, USA). The extraction was conducted using a 15-ml amber vial from Supelco (Bellefonte, PA, USA). Ten ml of sample solution containing 5 µl of 10^{-5} mol L^{-1} E2 was used for every experiment conducted to estimate the application of LPME pre-concentration on bioassay. The sodium chloride concentration of the sample was adjusted to 30% (w/v). Accurel Q3/2 polypropylene hollow-fibre (600 µm inner diameter, 200 µm wall thickness, 0.2 µm wall pore size) and a 0.8 cm stirrer bar were purchased from Membrana (Wuppertal, Germany) and Scienceware (Bel. Art Product, Pequannock, NJ, USA), respectively. Five µl of toluene and dichloromethane mixed solvent (50 : 50, v : v) was withdrawn into a microsyringe. The syringe needle tip was inserted into the hollow fibre, which was immersed into toluene for 10 sec for impregnation. The mixed organic solvent was then introduced into the fibre. The extraction was performed for 30 min under 700 rpm. After extraction, the syringe was taken out of the sample solution, and then a 2-µl volume of extract was injected into the GC injector port for chemical analysis. On the other hand, the extract was evaporated using nitrogen gas and the solvent was changed to 10 µl of DMSO for the bioassay.

2.3 *Extraction conditions of HF-LPME*

The HF-LPME parameters that were optimised included extraction time, fibre length, type of solvent, degree of agitation and concentration of sodium chloride. A single analyte (E2) was pre-concentrated to 5×10^{-9} mol L^{-1} in 10 ml of the sample. The effects of the various parameters on extraction were investigated by monitoring oestrogenic activity using the bioassay and the relative concentration to pre-assumed optimum conditions using chemical analysis. The various extraction times tested were 10, 20, 30, 40 and 50 min while the various hollow fibre lengths tested were 1, 1.5, 2, 2.5 and 3 cm. The effect of solvent on extraction efficiency was investigated by the addition of varying ratios of toluene and dichloromethane. The ratios tested were 1 : 0, 3 : 1, 1 : 1, 1 : 3 and 0 : 1 (v : v). The range of degree of agitation tested was 100, 300, 500, 700 and 1000 rpm. The effect of sodium chloride concentration on efficiency of extraction was investigated by addition of 5, 10, 20, 30 and 35 % (w/v) to 10 ml samples. After extraction, yeast two-hybrid assay and chemical analysis were performed.

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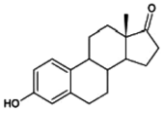
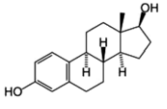
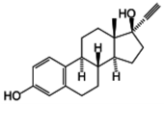
Table 1. Chemical characteristics and analytical parameters of target compounds.

Compound[a]	Structure	Log K_{oc}^{b}	Pa[b,c]	Solubility[b] (mg L^{-1})	EEF[b,d]	m/z[c]	Recovery (%)	LOD[e] (ng L^{-1})
E1		2.45–3.34	1.7×10^{-6}	0.8–12.4	0.01–0.2	218; 257; 342	92.05 ± 9.25	0.5
E2		3.10–4.01	1.4×10^{-7}	3.9–13.3	1.0	285; 416	77.91 ± 7.48	0.5
EE2		2.91–3.04	3.6×10^{-7}	4.8	1.0 – 1.7	196; 232	89.43 ± 7.98	5.0

Note: [a]E1 = estrone; E2 = 17β-estradiol; EE2 = 17α-ethinylestradiol; [b]References: [23,26–27]; [c]Pa = Vapour pressure; m/z = mass to charge ratio; [d]EEF = estradiol equivalent factor; [e]LOD = Limit of detection, corresponds to a signal-to-noise ratio of about 3 : 1.

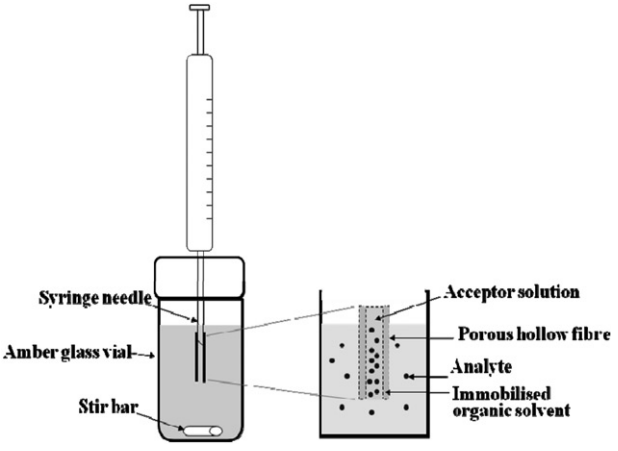


Figure 1. The experimental set-up of HF-LPME.

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2.4 *Yeast two-hybrid assay*

The yeast strain used in this study was *Saccharomyces cerevisiae* Y190 provided by Nishikawa [25]. The oestrogenic activity using yeast two-hybrid assay was determined by measuring the β-galactosidase activity. Yeast two-hybrid assay was carried out as follows. Yeast cells were pre-cultivated in SD (synthetic dropout, Trp^-, Leu^-) medium with 2% glucose and cultured for 16 h at 30°C and 180 rpm in a shaking incubator. The 50 µl of pre-culture was then incubated into fresh SD medium in a micro test tube including 25 µl of phosphate buffer and 2.5 µl of sample solution and incubated with shaking for 4 h at 30°C and 180 rpm. After 4 h, 150 µl of the aliquot was taken into a 96-well plate. Optical density at 595 nm was measured to determine cell concentration using an ELISA plate-reader (µQuant, Bio-tek Instruments, USA). The remainder was centrifuged for 5 min at 15,000 rpm to remove the medium and the cells were enzymatically digested by addition of 200 µl of 1 mg/mL Zymolyase 20T (MP Biomedicals, Ohio, USA) at 37°C for 15 min. The lysate was mixed with 4 mg/mL ONPG and reacted at 30°C for 30 min. After addition of 1 M Na_2CO_3 solution to stop the reaction, the absorbance at 420 nm and 570 nm were recorded to determine o-Nitrophenyl-β-D-Galoactopyranoside (ONPG, Sigma, St. Louis, MO, USA) concentrations and turbidity from cell debris, respectively.

Oestrogenic activity (U) was represented by the following equation [25]:

$$U = 1000 \times \frac{OD_{420} - (1.75 \times OD_{570})}{t \times v \times OD_{595}}$$

where t = reaction time with ONPG, v = volume of pre-incubated yeast cell, OD_{595} = optical density at 595 nm meaning the concentration of yeast, OD_{420} = optical density at 420 nm meaning the activation of β-galactosidase, OD_{570} = optical density at 570 meaning the disturbance of suspension. Relative oestrogenic activity was calculated with this following equation:

$$\text{Relative estrogenic activity} = \frac{U_{\text{test}}}{U_{\max \cdot \text{E2}}} \times 100$$

where U_{test} = oestrogenic activity of test solution, $U_{max \cdot E2}$ = maximum value of oestrogenic activity of 17-β estradiol.

The oestrogenicity of oestrogen mixture was also estimated using yeast two-hybrid assay. Oestrogen mixture includes E1, E2, and EE2. Oestrogen mixture was prepared in methanol and the spiked concentration was calculated using dose-response curve of each compound for comparison between the expected and observed oestrogenicity.

2.5 *GC-MS analysis*

GC/MS (QP 5050A-Shimadzu, Kyoto, Japan) coupled with an auto sampler (AOC-20S Shimadzu) was used for the chemical analysis. Chemical separation was carried out on a XTI-5 (Restek, Bellefonte, PA, USA) fused silica capillary column (length; 30 m, i.d.; 0.25 mm, film thickness; 0.25 μm). The GC program was set at 280 and 290°C for the injection and interface temperatures, respectively. Helium (99.9999%) was used as the carrier gas, with a flow rate of 1.0 mL min^{-1}. The oven temperature was set at 100°C for the initial 2 min, then ramped to 300°C at 10°C per min, and maintained at 300°C for 13 min. Target chemicals were analysed in the selected ion mode (SIM), with m/z (mass to charge ratio) values obtained in the full scan mode. Detailed information on the target chemicals and analysis parameters are provided in Table 1.

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3. Results and discussion

3.1 *Optimisation of HF-LPME on GC/MS analysis*

Chemical analysis using GC/MS was performed to determine the effect of the optimised parameters of HF-LPME on these analytical methods (Figure 2). The extraction time, type of solvent, degree of agitation, length of hollow fibre and salt content were selected as parameters that could potentially affect the efficiency of extraction. The relative concentration was defined as the percentage ratio of peak area of test solution to the maximum peak area of E2 with HF-LPME. One of the most important parameters affecting HF-LPME was the extraction time. The relative concentration of E2 to maximum concentration with pre-assumed optimum conditions increased to 68.4 up to extraction time of 40 min and decreased again at 50 min (61.8). A rapid increase in concentration was initially observed by a rapid partitioning between the aqueous sample and organic solvent, indicating that the LPME system required time to reach steady state between the two phases. However, it was observed that the analyte was slightly desorbed from the fibre for when long extraction times were employed.

Agitation of sample solution can accelerate efficiency of extraction. This is because the stirring rate allows the acceptor solvent to be continuously exposed to an aquatic sample and therefore faster agitation of the sample enhances the efficiency of extraction. In the case of single-drop LPME, the drop of acceptor solvent at the needle tip is commonly unstable during agitation [5]. However, HF-LPME system is capable of increasing the agitation rate, because the acceptor solvent is protected within the membrane. Different degrees of agitation ranging from 100 rpm to 1000 rpm was investigated to determine the optimal agitation. The relative concentration of E2 was highest at 700 rpm.

In the selection of an appropriate fibre length, various length of hollow fibre was compared and the relative E2 concentration was remarkably increased with 2.0 cm of fibre length. However, fibre length of 1 cm couldn't contain all solvents and some of the solvent

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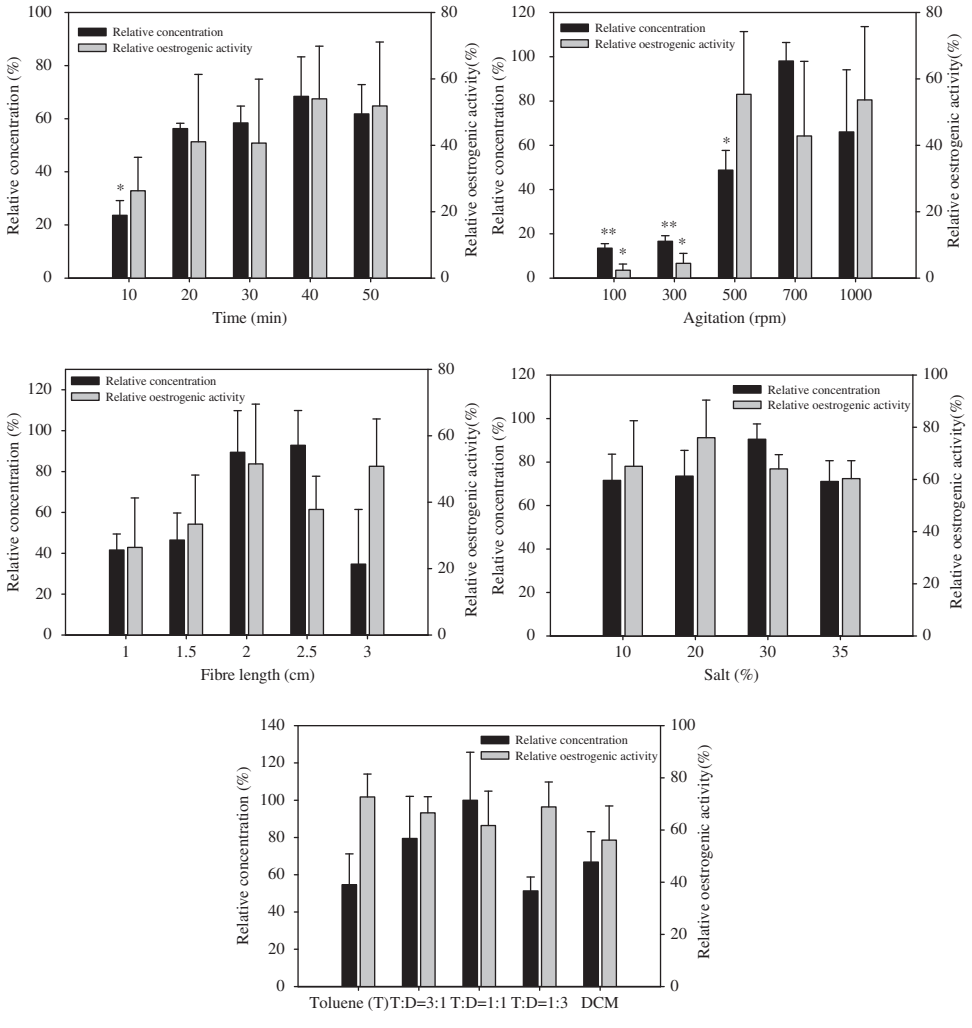

Figure 2. The effect of various parameters of HF-LPME pre-treatment methods on Yeast two-hybrid assay and GC/MS chemical analysis (mean ± standard error). Relative concentration indicates % ratio of peak area of test solution to the maximum peak area of E2 with HF-LPME. Relative oestrogenic activity indicates the % ratio of oestrogenic activity of test solution to the maximum value of oestrogenic activity of 17β-estradiol with HF-LPME. The asterisk (*) and (**) indicate $p < 0.05$ and $p < 0.01$, respectively.

were released due to its volume capacity. Though E2 concentration was increased with 2.5 cm of fibre length, it was mechanically more difficult to perform the extraction since agitation made the fibre at the needle tip unstable. From these results, 2.0 cm fibre length was selected as the optimum fibre length.

The effect of salt on the efficiency of extraction was investigated by adding varying percentages of sodium chloride to water samples within a range of 5, 10, 20, 30, and 35% (w/v). Addition of sodium chloride can reduce the solubility of the analyte in an aqueous sample and thereby increase the partitioning of the analyte into the acceptor solvent.

The salt concentration was reported to be most effective at 30% (w/v), indicating the highest relative E2 concentration.

Finally, the selection of the extraction solvent was determined. There are three factors that are recommended to be considered during the selection of an acceptor solvent for hollow fibre LPME [5]. Firstly, since the pores within hollow fibres should be completely filled with an appropriate solvent, the solvent must be compatible with the fibre. Secondly, the solvent should be immiscible with water. Finally, the acceptor solvent must be appropriate for the chemical analysis or bioassay. To this end, toluene was easily immobilised on the hollow fibre and its solubility in water was reported to be low at 0.53 g/L. Also, dichloromethane (DCM) has been previously used in the extraction of oestrogenic compounds. Thus, toluene and DCM and a mixture of both solvents with different volumes were used as the extraction solvent; Toluene only, Toluene : DCM = 3 : 1, Toluene : DCM = 1 : 1, Toluene:DCM = 1 : 3, and DCM only. According to the results, the most effective extraction solvent on chemical analysis using GC/MS was determined as a volume ratio of 1 : 1 in toluene and DCM.

From these results, the optimised HF-LPME conditions for GC/MS analysis were summarised as follows: 40 min extraction time, Toluene : DCM = 1 : 1 (v : v) as the extraction solvent, 700 rpm stirring rate, 2.0 cm fibre length, and 30% (w/v) salt concentration.

3.2 *Optimisation of HF-LPME on bioassay*

The parameters affecting HF-LPME were also optimised based on the highest oestrogenic activity obtained from the yeast two-hybrid assay (Figure 2). In order to evaluate the efficiency of HF-LPME on EDCs extraction for bioassay monitoring of oestrogenic activity, we used E2 as a target compound. In a similar manner to the optimisation of parameters of the chemical analysis, this study was performed for five parameters. The relative oestrogenic activity was found to have increased from 26.3 to 54.0 when the exposure time increased from 10 to 40 min; the relative oestrogenic activity at 50 min was similar to that at 40 min. Therefore, 40 min of extraction time was selected as optimal for this study.

An agitation speed of 100 rpm and 300 rpm showed extremely low oestrogenic activity of 2.4 and 4.4, respectively, in contrast to an agitation speed of 500 rpm yielding an oestrogenic activity of 55.4. On the other hand, fast stirring at 1000 rpm was mechanically difficult to control because air bubbles were generated and the hollow fibre was easily detached from the tip of the syringe; the air bubbles attached to the hollow fibre could promote solvent evaporation [5]. Therefore, 500 rpm was selected as the optimum degree of agitation.

Relative oestrogenic activity was 26.4 at 1.0 cm of fibre length. Oestrogenic activity was increased to 51.5 up to a fibre length of 2.0 cm and then decreased to 37.8 at 2.5 cm. From these results, 2.0 cm fibre length was selected as the optimum fibre length.

The effect of salt on the efficiency of extraction was investigated by adding varying percentages of sodium chloride to water samples. The results indicated that the most effective salt concentration was 20% (w/v) of salt, indicating the highest relative oestrogenic activity.

Toluene and DCM and a mixture of both solvents with different volumes were used as the extraction solvent. In the present study, no significant differences between the extraction solvents were found. However, the relative oestrogenic activity showed

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a tendency to decrease with an increase in DCM volume. Moreover, when 100% concentration of DCM was used as the acceptor solvent for extraction, a bubble was easily formed in the hollow fibre. Therefore, DCM was not considered to be a suitable extraction solvent in hollow fibre LPME for bioassay experiments. Based on this result, toluene was selected as the optimised solvent in this study.

According to the results of the present study, the degree of agitation and the extraction time were found to be the most influencing parameters in HF-LPME. Overall, the optimised conditions of HF-LPME for a yeast two-hybrid assay were as follows: 40 min of extraction time, toluene as the extraction solvent, 500 rpm stirring rate, 2.0 cm fibre length, and 20% (w/v) salt concentration.

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3.3 *Evaluation of oestrogenic activities of EDCs using a yeast two-hybrid assay*

All relative concentrations obtained by the GC/MS analysis versus the relative oestrogenic activity obtained by the yeast two-hybrid assay with different conditions of parameters were compared (Figure 3). The values of parameters suitable for chemical analysis and bioassays were selected; that is, the results with high relative concentrations in GC/MS analysis showed substantially higher relative oestrogenic activity in bioassays. In the case of fibre length, extraction time, and degree of agitation, the variation of relative oestrogenic activity and concentration were higher than any of the other two parameters. This inferred that these three parameters were the major contributors to HF-LPME and therefore have to be controlled for effective extraction. Although the solvent type caused remarkable changes for chemical analyses, it did not contribute significantly toward the

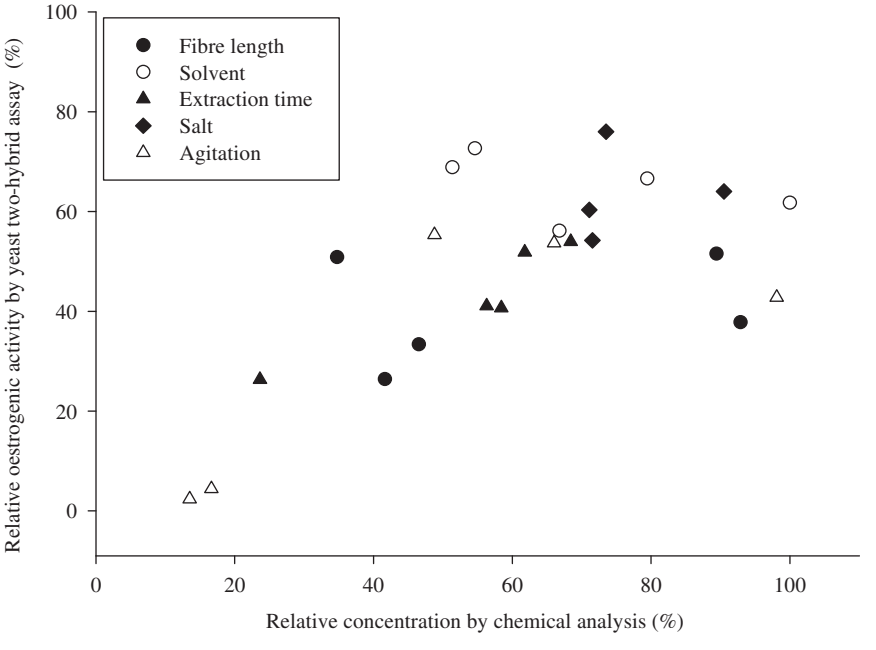

Figure 3. Comparison between chemical analysis and bioassay with hollow fibre LPME.

results obtained from bioassays. Lastly, it was determined that the salt content did not contribute significantly to either chemical analyses or bioassays with HF-LPME.

The *in vitro* assay like yeast two-hybrid is simple, speedy and easy to interpret than *in vivo* assay. In addition, the amount of target compounds required for the yeast two-hybrid assay was only 2.5 µl for each test tube. The yeast two-hybrid assay is a suitable method for the samples with small volume, which could be well associated with optimised HF-LPME techniques.

3.4 *Comparison with other pre-concentration techniques*

Oestrogenic activities of E2 at different concentrations were investigated in order to estimate the applicability of the optimised parameters in this study (Figure 4). The concentration was normalised according to the average recovery rate of each pre-treatment method, 20% for HF-LPME and 60% for SPE, respectively. Oestrogenic activity with HF-LPME was similar with SPE pre-treatment at normalised concentration. Furthermore, higher oestrogenic activity was shown at the concentration of 10^{-6} mol L^{-1}. HF-LPME showed identical shape of dose-response curve whereas the shape of response curve was disordered after SPE pre-treatment. This result indicated that HF-LPME was appropriate for the pre-treatment method for yeast two-hybrid assay if the normalised factor was considered.

A mixture of oestrogens that included E2, EE2 and E1 were pre-treated using HF-LPME to find out the mixture effect such as synergistic, antagonistic or independent effect and the oestrogenic activity was compared with SPE (Figure 5). The results of concentration-response curve plotted by the normalised cell proliferation from the yeast two-hybrid assay towards oestrogenic compounds including 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and estrone (E1) were used for mixture experiment.

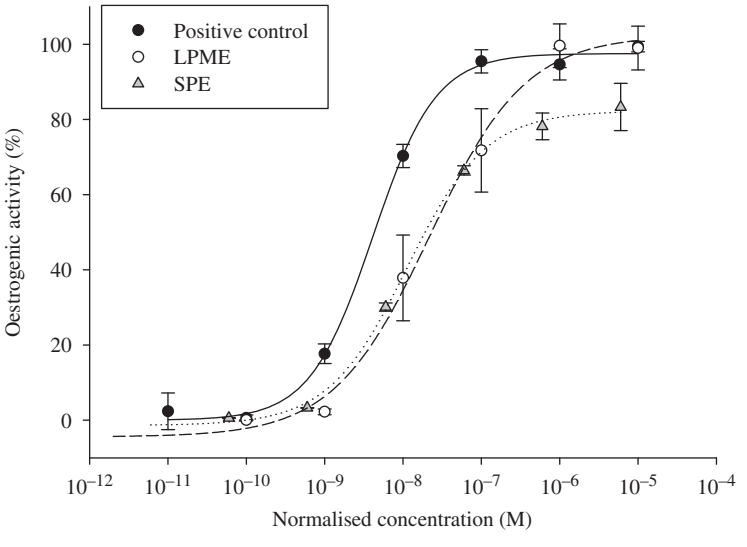

Figure 4. Relative oestrogenic activity of E2 in yeast two-hybrid assay with HF-LPME (○) and conventional solid phase extraction (▲) to control condition spiked with E2 (●).

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The parameters of regression equation and the EC_{50} values of each oestrogen were calculated by the sigmoid best-fit curve (Table 2). E2 and EE2 showed much higher relative potency values than E1. Normalised concentration was considered for expected oestrogenic activity with both pre-treatment methods. The oestrogenic activity of mixture using solid phase extraction (SPE) showed higher values in comparison with HF-LPME. This higher oestrogenic activity of SPE was due to the relative higher volume of acceptor solvent than HF-LPME. Related result was observed by López-Blanco *et al*. [28]. The volume of acceptor solvent had an effect on the efficiency of single drop micro-extraction. The volume of extraction solvent of SPE in this study was 1000 times higher than HF-LPME, because it was mechanically impossible to extract SPE column with micro-volume of acceptor solvent. In spite of the small volume of acceptor solvent in HF-LPME, the normalised oestrogenic activity with yeast two-hybrid assay was well matched with expected oestrogenicity. According to other studies [29–30], it was demonstrated that the mixture of oestrogens including E2 and EE2 were shown to act together in an additive manner and concentration addition model, based on no interaction

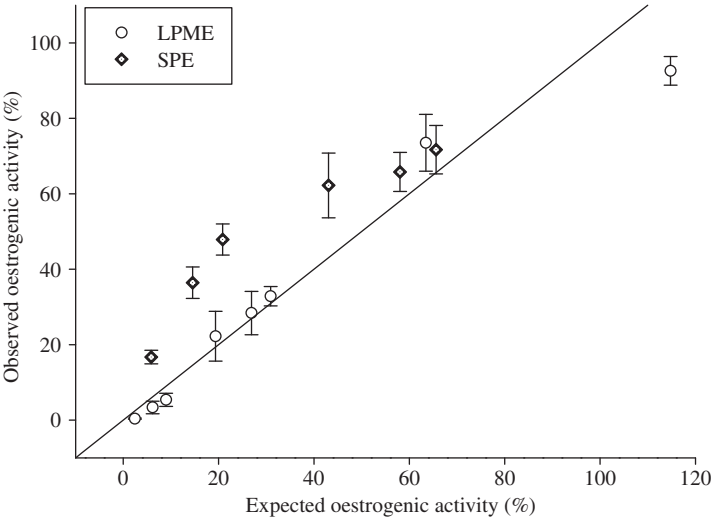

Figure 5. Comparison of relative values between observed and expected oestrogenic activity of chemical mixtures (e.g. E1, E2 and EE2) using yeast two-hybrid assay with HF-LPME and solid phase extraction (SPE) to control condition. The line indicates the equality.

Table 2. Parameters of concentration-response curves, the equation: $y = a/[1 + (x/x_0)^b]$, toward 17β-Estradiol, 17α-ethinylestradiol, and Estrone.

Parameter	17β-Estradiol (E2)	17α-ethinylestradiol (EE2)	Estrone (E1)
a	97.52	107.72	81.62
b	−1.08	−1.06	−1.47
x_0	4.11×10^{-9}	6.32×10^{-9}	1.38×10^{-8}
r^2	0.99	0.99	0.99
EC50 (M)	4.45×10^{-9}	5.52×10^{-9}	1.91×10^{-8}
Relative potency	1	0.81	0.23

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in combination. This may be proper to predict its real combined effect. Therefore, the results from HF-LPME are quite well matched to the model and showed more accurate prediction of combined effect for the mixture of oestrogens.

In this study, the results indicated that the fibre length, extraction time and degree of agitation were the major contributing parameters to HF-LPME and should be controlled for effective extraction. Although the type of solvent caused remarkable changes for chemical analyses, it did not affect the efficiency of the bioassay. In comparison with SPE, HF-LPME showed lower oestrogenic activity because of the difference of extraction volume. However, HF-LPME showed more accurate oestrogenicity of E2 after considering the normalised concentration and good correlation rate of expected and observed oestrogenicity in oestrogen mixture. Generally, the HF-LPME method that applied for small volume samples has been confirmed by chemical analysis using the GC/MS or LC/MS system. However, the detecting of oestrogenicity using bioassay is required for oestrogen chemicals causing endocrinal disruption because the oestrogenic activity is an index of real effect on biota. Furthermore, the measurement of whole oestrogenic activity for small volume sample is suitable with yeast two-hybrid assay and well associated with the HF-LPME technique. Consequently, bioassays such as yeast two-hybrid assay could be easily, rapidly and inexpensively conducted with small volumes of sample using HF-LPME. However, further study is needed to increase the absolute recovery rate in comparison with positive control.

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