Video Article

A Simple Method for Automated Solid Phase Extraction of Water Samples for Immunological Analysis of Small Pollutants

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Abstract

A new method for solid phase extraction (SPE) of environmental water samples is proposed. The developed prototype is cost-efficient and user friendly, and enables to perform rapid, automated and simple SPE. The pre-concentrated solution is compatible with analysis by immunoassay, with a low organic solvent content. A method is described for the extraction and pre-concentration of natural hormone 17β-estradiol in 100 ml water samples. Reverse phase SPE is performed with octadecyl-silica sorbent and elution is done with 200 µl of methanol 50% v/v. Eluent is diluted by adding di-water to lower the amount of methanol. After preparing manually the SPE column, the overall procedure is performed automatically within 1 hr. At the end of the process, estradiol concentration is measured by using a commercial enzyme-linked immune-sorbent assay (ELISA). 100-fold pre-concentration is achieved and the methanol content in only 10% v/v. Full recoveries of the molecule are achieved with 1 ng/L spiked de-ionized and synthetic sea water samples.

Video Link

The video component of this article can be found at https://www.jove.com/video/53438/

Introduction

Sample preparation is an important step in any analytical process. In particular, removal of matrix effects, diminution of interferences, and enrichment of the analyte are necessary to obtain precise results and reach low limits of detection. Endocrine disrupting compounds (EDCs) are of particular concern due to their action on the living organisms even when present at very low levels in the environment. The natural hormone 17β-estradiol is present on the EU water pollution Watch List and prone to be added to the list of priority substances regulated under the European Water Framework Directive. Solid phase extraction (SPE) is commonly applied for the analysis of small pollutants in water, with both chemical [1-5] (chromatography, mass spectrometry) and immunological [6-9] detection methods. The latter gained interest in the field of environmental monitoring, as immunoassays are available in large variety of formats, are specific to the target analyte, and reach low limits of detection.[6, 7, 10, 11] Various enzyme linked immunosorbent assays (ELISA) are commercially available and enable to analyze multiple samples at once on a multi-well plate. The procedure consists in successive reaction steps that can take a few hours. The final product of reaction can be detected optically to determine the concentration of the target molecule based on a calibration curve.

Classical SPE procedures include sorbent pre-conditioning, sample extraction, washing, elution, and concentration by evaporation of the eluent. The solvent used for dilution of this extract is chosen depending on the detection method. For immunological methods, the amount of organic solvent influences strongly the sensitivity of the method.[12]

In addition to the recovery and the pre-concentration performances, the method also needs to be simple and cost efficient. Automation of the procedure helps to reduce human-related errors. In our previous work [13] we introduced our prototype for automated SPE, and our method was applied to the analysis of the natural hormone 17β-estradiol in sea water samples. With the present video we would like to highlight the technical advantages of our method compared to traditional off-line and on-line SPE, and its particular compatibility with detection by immuno-reactions. We describe the protocol applied to water samples for the detection of 17β-estradiol. SPE is performed with octadecyl-silica (C18) sorbent phase and elution is performed with diluted methanol.

Protocol

Note: The following protocol describes the SPE performed on 100 ml water sample with C18 sorbent and elution with 50% v/v methanol. The enriched sample is diluted to reach 10% v/v methanol before analysis with an enzyme linked immunosorbent assay (ELISA) kit.

1. Preparing the Reagents

1. Prepare the water samples
    1. Prior to any other step, filter each 100 ml water sample with 0.2 µm pore size filters.
    2. Spike the sample with desired concentration by diluting appropriate volume of reference solution into a volume of water. For example, prepare 100 ml of water sample with 100 ng/L of E2 by diluting 3.3 µl of E2 reference solution at a concentration of 300 µg/L. Dilute this solution ten times to get a sample spiked with 10 ng/L E2. Dilute this latter another ten times to obtain a sample of 100 ml with 1 ng/L E2.
    3. Place the filtered sample (unmodified or spiked) in a glass bottle with GL45 thread. Use the sample on the same day. Take a small fraction to be analyzed with the ELISA to estimate initial concentration.
2. Prepare 300 µl of eluent by diluting methanol in de-ionized (di-) water to 50% v/v in a tight tube.

2. Preparation of the SPE Column

1. Prepare a 20 mg/ml suspension of octadecyl-silica sorbent particles by adding first 1,600 µl of methanol and then 400 µl of di-water to 40 mg of reverse phase sorbent. Tightly close the lid and agitate with a vortex.
2. Installing the bottom membrane in column
    1. Select one Nylon membrane with pore size 11 µm and place it on a double layer of anti-dust tissue. With a 3 mm diameter punch, cut two small parts in the membrane.
    2. Grab one of the small membranes with a flat-end filter forceps and place it on one side of the column.
    3. Screw the flat-bottom connector with the tube and tighten. The membrane is now in place and the sorbent can be added securely. Draw an arrow on the body of the column pointing towards the end where the membrane was placed.
3. Preparing the packed sorbent column
    1. Secure the column on the holder with the arrow pointing towards the bottom. The column must be set as vertically as possible.
    2. Attach an empty 10 ml disposable syringe to the end of the tube of the column, by using the Luer-Lock connectors.
    3. Prepare a micro-pipette with a 20-200 µl tip, set the volume to 100 µl. This pipette tip format is adapted to the size of the sorbent column to be prepared. The procedure would be more difficult with larger pipette tips such as the ones used with 1 ml pipettes.
    4. Agitate the sorbent suspension with a vortex, and rapidly pipet 100 µl in the center on the column. While injecting, aspirate gently all the pipetted solution trough the membrane by using the syringe with the other hand. At this stage, the solution filling the syringe must be clear of particles, and a particle bed can eventually be observed in the column.
    5. Repeat this process 2 more times by agitating the stock suspension between all pipetting steps to ensure homogeneous suspension of the particles in the solution. The resulting column contains 6 mg of sorbent.
    6. When the 300 µl of suspension have been loaded and dried by aspirating with the syringe, keep the syringe in position and place the second Nylon membrane on the top by using the forceps with the other hand.
    7. Screw down the second connector with tube and dispose the syringe. The SPE column is ready for use.

3. Preparing the System

1. Tighten the SPE column on the device by using the Luer-Lock connectors. The arrow must be pointing to the same direction as the one shown on the device.
2. Connect the bottle containing the sample to the device by screwing the provided GL45 safety cap on it.
3. Load 200 µl of eluent in the 'Eluent' reservoir.
4. Load 800 µl of di-water in the 'Dilution' reservoir.
5. Place a bottle at the waste outlet to collect the processed water during extraction step. The format is not important but the volume needs to be sufficiently large with regard to the sample volume.
6. Place a small vial at the sensor outlet with the minimum volume capacity of 1.5 ml. A maximum volume of 1 ml will be too small as bubbles will form at this outlet when collecting enriched eluent and dilution buffer.
7. Verify the pressure regulator is in closed position by turning it manually, reverse-clockwise until further movement is not possible.

4. SPE with the Prototype

1. Switch on the prototype by pressing the button on the back.
2. Preparing the user interface and selecting the program
    1. Open the user interface. Select the communication port of the computer to which the device is connected from the list, and click the button 'Next'.
    2. Enter the values 580 for *pset* and 30 for *dpset*. The pump will adjust to maintain pressure in the pressurized system and reservoirs at 580 ± 30 mbar when running.
    3. Select the automated mode.
    4. In the automated mode corner, load the program file in the box 'configuration file path'.
3. Adjusting the pressure regulator
    1. Start the pump.
    2. Turn manually the pressure regulator until the value read for *preg* is inferior but close to 320 mbar.
    3. Stop the pump.

4. Starting the SPE procedure
    1. Press 'start' in the automated mode corner. The pump will switch on and the extraction, elution and dilution steps will automatically follow one another. The whole procedure for a 100 ml sample is performed in 50 min.
    2. Verify the value of preg. It must be in the range 320 - 350 mbar during the extraction step to ensure an optimal flow-rate.
    3. Close the small vial and store at 4-5 °C in the dark until analysis. Perform analysis in the following 30 hr to prevent degradation of the analyte.
    4. Dispose of the processed water.

5. Cleaning the system
    Note: After each extraction procedure the system needs to be cleaned to prevent cross-contamination.
    1. Prepare a 10 ml solution of methanol 70% v/v in a GL 45 glass bottle.
    2. Unplug the SPE column and plug the tubing with connectors.
    3. In the automated mode, select the 'cleaning' file and start it with same pressure settings.

5. Detection of Estradiol Concentration with ELISA

1. Prepare the calibration samples with concentrations as indicated in the protocol provided with the ELISA kit that is used. Prepare one calibration set with the same matrix as the sample, and one calibration set with methanol 10% v/v.
2. Dispense the necessary amount of sample in the wells on the plate, according to manufacturer's instructions. Use calibration samples, unmodified and spiked water samples that were filtered but not processed with SPE, and enriched samples in methanol 10% v/v. Use 3 wells per sample to reduce the error associated with the assay.
3. Follow the indications of the protocol that is provided with the kit for the addition of reactants, incubation time, washing and further reaction with the enzyme.
4. Read the optical signal in each well according to the kit manufacturer's instructions with a plate reader instrument and collect the data.
5. Use the software of the plate reader instrument to fit the calibration curves and determine the E2 concentration in the initial sample with same matrix, and in the enriched samples with methanol 10% v/v.

Representative Results

Reproducibility of sorbent packing was evaluated by drying and weighting the pipetted sorbent in glass vials and the result is shown in **Figure 1**. Reproducibility of the time of injection was tested for 100 ml samples, as shown in **Figure 2**. Concentration in initial and pre-concentrated spiked samples were determined by using a commercial ELISA kit for 17β-estradiol and are shown in **Figure 3**.

The proposed procedure involves the user for the preparation of the sorbent phase (**Figure 1**). The sorbent particles are held between two membranes for mechanical stability and are densely packed by the pressure applied with the syringe while pipetting the suspension in the column. The resulting column contains an optimized amount of 6 mg of the chosen sorbent with only 6% error on this value. This step also acts as sorbent conditioning, as the suspension is prepared in appropriated solvent conditions.

After preparing and installing the SPE column on the system, and loading the solutions in the appropriate reservoirs, the pre-concentration procedure is fully automated and requires in total less than 1 hr for a 100 ml sample (**Figure 2**). Extraction is performed in 40 ± 8 min. Only two parameters are still influenced by the user, the preparation of the packed sorbent and the setting of the flow-rate. The first would be solved by applying large scale methods for column fabrication. The second is related to the manual adjustment of the pressure regulator, which determines the pressure value used to drive the solutions through the system. This source of error would be eliminated by implementing an electronic pressure regulator.

Regarding performances, the pre-concentration factor achieved is 100. First, 500-fold pre-concentration is done by eluting the extract from the sorbent with 50% v/v methanol. Then a 5-times dilution is performed by adding di-water. This dilution reduces the solvent content and preserves the immunoassay sensitivity. When looking at the calibration curves of the ELISA (**Figure 3A**), it is clear that the methanol ratio in the enriched sample does not affect the sensitivity of the immunoassay. A representative result of pre-concentration is shown in **Figure 3**. The method was applied to di-water and artificial sea water samples spiked with 1 ng/L of 17β-estradiol. While the sample concentrations are below the limit of detection, the pre-concentration method successfully brings those samples in the range of the assay (30 - 30,000 ng/L). The recoveries were obtained by comparing the final concentration with theoretical spiked concentration. Recoveries of 128% ± 22% and 107% ± 6% were calculated for di-water and artificial sea water respectively (**Figure 3B**).

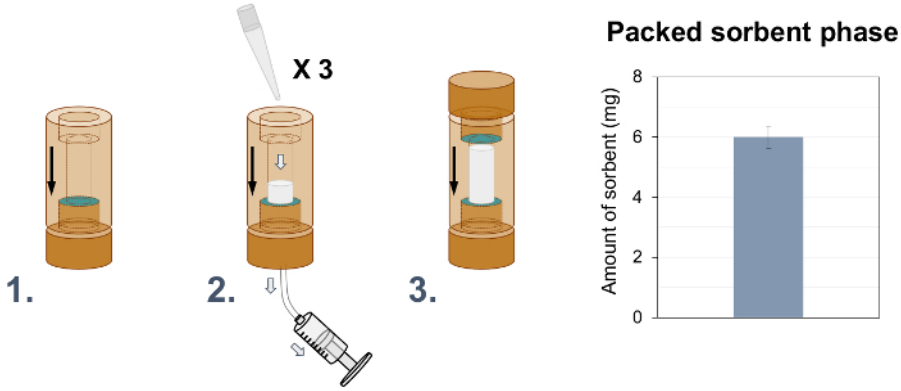

Figure 1. Illustration and reproducibility of the method for sorbent packing. There are three steps: securing the first Nylon membrane with the first connector, injecting the sorbent suspension, and closing the column by inserting the second membrane and connector. The resulting column contains 6 mg of sorbent with 6% standard deviation (n = 6 prepared and weighted columns). Please click here to view a larger version of this figure.

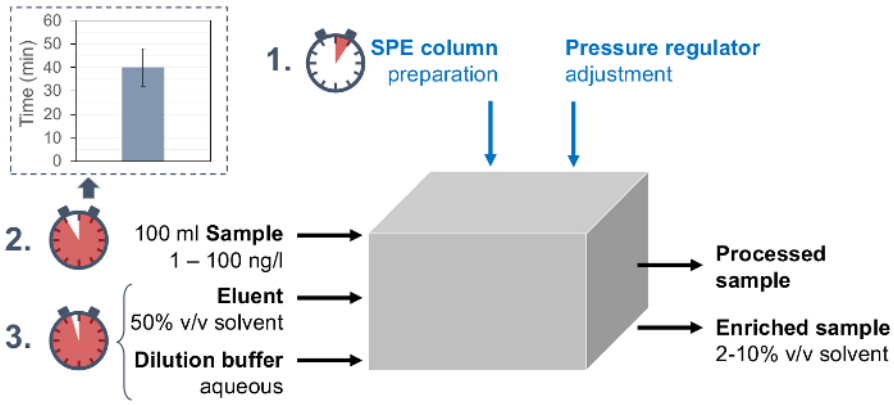

Figure 2. Step-by-step illustration of the procedure with reproducibility of time for sample injection. The solution inputs are loaded in the reservoirs or bottle. The two outputs are the processed sample (waste) and the enriched sample, which is compatible for analysis by immunoassay with low solvent content. The overall procedure is automated and takes slightly less than 1 hr (n = 31 with standard deviation). The influence of the user on the flow-rate is highlighted with blue characters. Please click here to view a larger version of this figure.

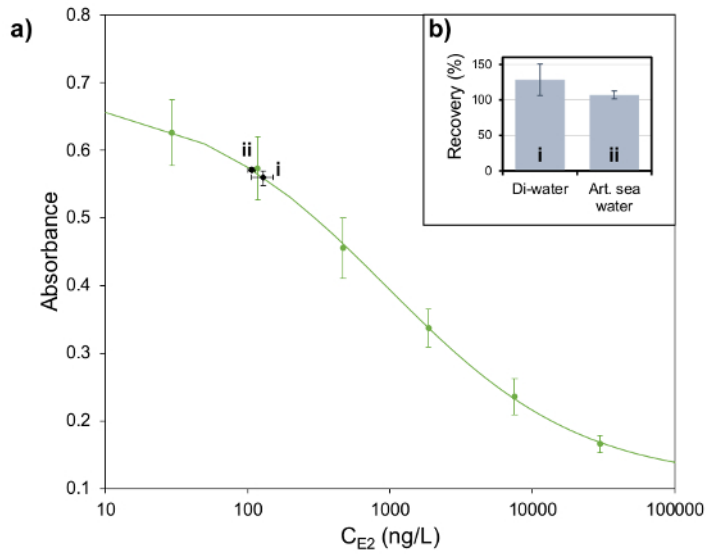

Figure 3. Results of the pre-concentration of E2 and analysis by ELISA. (**A**) Calibration curves of the ELISA and points measured for 1 ng/L spiked di-water (i) and artificial sea water (ii) after enrichment. (**B**) Recoveries obtained for 1 ng/L spiked di-water (i) and artificial sea water (ii) samples after enrichment (n=4). The error bars are standard deviations arising from the number of replicates and the 3 wells on the ELISA plate that were used to determine the concentration in each sample. This figure has been modified from ([13]). Please click here to view a larger version of this figure.

Discussion

A new method for the preparation of water samples followed by analysis using immunoassay was proposed. The instrument enables to perform solid phase extraction in an automated and user-friendly way.

The filtration of the water sample prior to its injection into the system is critical. Any particulates still present in the solution would potentially cause clogging of the fluidic network and obstruct the SPE column. Another important step is the preparation of the SPE column. The amount of particles in the column is critical to achieve the best performances possible. Special care must be taken when preparing the suspension of sorbent particles, to avoid agglomeration of the particles. This is achieved by adding first the solvent fraction to the dry sorbent, and then the di-water fraction. Then, during the packing step, it is important to mix the suspension well while aspirating the 100 µl with the pipette. At the end of the SPE procedure, properly cleaning the system is critical. Firstly, it prevents cross-contamination when working with different samples, and secondly it avoids the risk of bio-contamination of the instrument when it is not used.

As discussed in the introduction, the use of immuno-detection methods for analysis of small pollutant molecules in the environment is expanding. Those methods reach limits of detections in the low ng/L levels [7, 11] and have the advantage of being very specific. Such methods are used in combination with chemical methods, mostly mass-spectrometry related techniques. [14, 15] The latter do not restrict the use of organic solvent and benefit from automated SPE systems that enable reaching the required high pre-concentration factors. In comparison, immunoassays are more sensitive to the assay buffer composition and lack adapted sample preparation techniques to facilitate the process. Our module is dedicated to the analysis of small molecules by immunoassay.

With our automated method, a 100-fold enrichment of the sample is achieved and permits to detect the analyte in the ELISA concentration range. If those pre-concentration performances seem low compared to traditional SPE, they perfectly match the requirements for immuno-detection. Moreover, the instrument has a small footprint (25 cm x 15 cm x 10 cm) and low-cost compared to typical manual or automated SPE setups.[13] If needed, the throughput for multiple sample analysis can therefore be increased by using a few devices in parallel. Another possibility would be to design a method for smaller volumes of sample and eluent, which would reduce the time needed for the procedure. Some other limitations were discussed through the description of the results. There are still two steps where the user is involved, both linked to the degree of development of this prototype and would be solved by making one step further towards a commercial device. The packing of the sorbent phase is done manually. The method was however shown to be easy and reproducible. We are confident that disposable columns could be produced if the system were produced on a higher scale.

In summary, we have described a new method to perform SPE on a compact, automated device. We have demonstrated its potential for analysis of water samples by immunoassay through the description of an extraction and pre-concentration method applied to detection of 17β-estradiol by a commercial ELISA kit. The method is user friendly and cost effective, and could in the future be applied in-line with biosensors (work in progress). We expect our platform will enable to perform similar solid phase extraction procedures on more complex sample matrices of high relevance for the monitoring of EDCs, such as food or urine. We are convinced our system will support the application of established and upcoming immuno-detection methods in the field of environmental analysis.

Disclosures

The authors have nothing to disclose.

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