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Sample Pretreatment Protocol for Female Steroid Hormones

Japan EnviroChemicals, Ltd.

JEC ELISA Kits for Female Steroid Hormones

JEC ELISA Kit	Target Compound	Dynamic Range	IC50 (B/B0=50%)
E1	E1	50ng/L - 5000ng/L	300ng/L
E2	E2	50ng/L - 1000ng/L	150-200ng/L
EE2	EE2	50ng/L - 3000ng/L	200ng/L
ES	Total estrogens (E1, E2, E3)	100ng/L - 3000ng/L	300ng/L

Essential Reagents/Materials for Pretreatment

1. Disposable test tubes (e.g. IWAKI, item No. 9831-1207)
*Be sure to use disposable tubes to avoid adsorption.
2. Glass fiber filter
   (1) ADVANTEC (Toyo Roshi, JAPAN)
   http://www.advantec.co.jp/english/contact/index.html.
   FILTER PAPER GLASS FIBER, GS-25,47mmφ
   CodeNo.36481047
   100quantity／box

3. Filtering equipment

Filtering Equipment

Filtering Equipment (assembled)

Vacuum Pump

Filtering Setup

4. Micropipettes (20µL - 200µL and 100µL - 1000µL, e.g. Gilson Pipetman P-200, P-1000) and tips (e.g. ICN Superpack 96NS)
5. Multichannel pipettes (50µL - 300µL e.g. LabSystems Finnpipette Digital 8-channel Pipettor) and tips (e.g. ICN Superpack 96NS)
6. Microplate reader (450nm wavelength) (e.g. TECAN Sunrise Remote)
7. Stop watch
8. Strip ejector (e.g. COSTAR, No.2578)
9. Solvent: Methanol, Hexane, Dichloromethane (HPLC grade)
10. C18 Solid phase extraction cartridge
   (1) J.T. Baker Inc. (NJ, USA):
   http://www.jtbaker.com/
   BAKERBOND spe Octadecyl (C18) Disposable Extraction Columns
   6ml Solid Phase Extraction Columns, 500mg per column
   *Code No.7020-06 *30quantity/box
   (2) Waters Corporation (MA, USA):
   http://www.waters.com/WatersDivision/
   Sep-Pak C18, 500mg/6cc
   *Code No.043395 *30quantity/box
11. Aminopropyl (NH2-propyl) Solid phase extraction cartridge
   (1) Waters Corporation (MA, USA)
   http://www.waters.com/WatersDivision/
   Sep-Pak Plus NH2 Cartridges
   360mg/cartridge
   *Code No. WAT 020535 *50quantity/box

*Vacuum manifold is useful for higher sample throughput.

Vacuum Manifold with SPE C18 Cartridge




1. Sample Filtration

Filter raw water sample (e.g. 1 liter) through the specified glass fiber filter (1μm pore diameter). To save time, suctioning with a vacuum pump is recommended. Change filters if necessary. As a rule of thumb, one filter can process 300-500ml of treated water (effluent) or 100-200ml of raw wastewater (influent). If an influent sample contains a large quantity of suspended matter, centrifuge the sample to obtain the supernatant for filtering.

If there remains sediment on the filter, pour MeOH to extract the analyte from the solid and add the eluant to the filtrate.

Make sure the amount of MeOH dose not exceed 1% of the total volume of the filtrate. i.e. For 1liter of filtered sample, the amount of MeOH should be less than 10mL.

Confirm the pH of the filtrate is between 5 and 8. If pH is out of this range, add acid or base to adjust pH.

Follow the solid phase extraction procedure: Method A or Method B. Method A, a simpler protocol, is applicable in any influent and effluent except E1 in influent. E1 in influent is best prepared with Method B. Method B does not affect the quantitation of E2 nor EE2 and you may use Method B for all the samples. Below is the matrix of target compounds and the recommended protocol.

	Target Compound			
	E1	E2	EE2	E1+E2+E3
Influent	Method B	Method A or B	Method A or B	Method B
Effluent	Method A or B	Method A or B	Method A or B	Method A or B

2. Solid Phase Extraction: Method A

(1) Protocol

1. Rinse a C18 cartridge with 5 ml of methanol and then 10 ml of distilled water (Preconditioning). To save time, suctioning with a vacuum pump is recommended. Flow rate, then, should not exceed 20ml/minute.
2. Then, pour the filtrate, prepared in Section 1 (Sample Filtration), through the C18 cartridge at a flow rate, no faster than 20ml/minute.
3. Wash the cartridge with 5ml of distilled water (up to 20ml/minute). Keep suctioning for about a minute to dry the cartridge. Then, wash the cartridge with 5ml of hexane (up to 20ml/minute).
4. Elute the analyte with 5ml of dichloromethane at a rate of 3ml/minute.
5. Evaporate the solvent with nitrogen gas.

   The typical setup is shown in the picture. Nitrogen gas, or (only if not available) compressed air, is supplied through pasteur pipettes into 10ml tubes containing eluted sample. Temperature is controlled at 40 - 50 degrees C with a water bath to accelerate evaporation.

6. Add 100% methanol to the residue and stir the mixture with a vortex. Terminate the mixing and pour distilled water to adjust the content at 10% methanol (v/v).

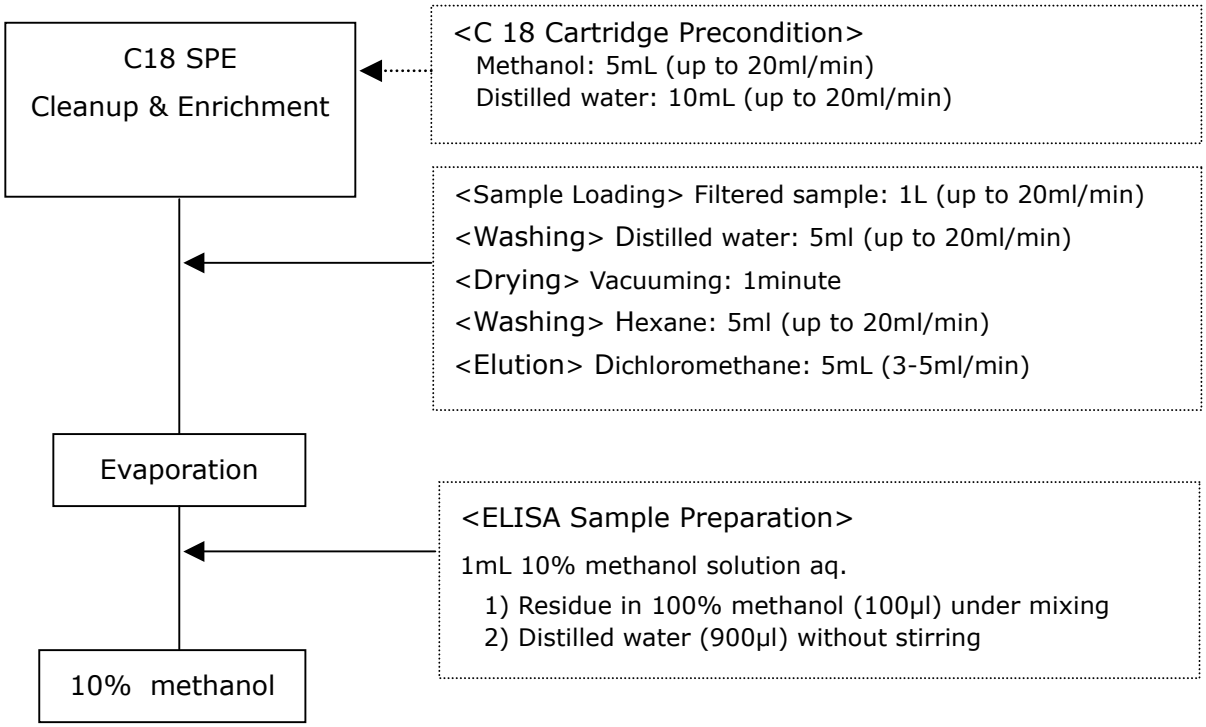
   If the initial amount of sample is 1liter, 1ml of the resulting 10% MeOH solution means 1000 fold concentration. Also prepare a 10-fold dilution from this concentrate so that the absorbance of either sample will fall in the dynamic range of the kit. Further dilutions may be necessary for some samples such as untreated wastewater.

   Example:

Sample Matrix	Typical Concentration	Recommended Sample Series
Treated water (effluent) River water	1ng/L (E2)	- 1000-fold concentration - 10-fold dilution of the above concentrate
Raw wastewater (influent)	10-100ng/L (E1, E2)	- 1000-fold concentration - 10-fold & 100-fold dilutions of the above concentrate

   If most of the residue remains undissolved, evaporate the 10% methanol solution again with nitrogen gas. Add 10µL of 100% DMSO to the obtained residue and stir the mixture with a vortex. Then, add 100µL of 100% MeOH, 10 times of the volume of DMSO, terminate the mixing, and add distilled water until the total volume amounts to 1000µL (1ml). The composition of the resulting solution should be 1%DMSO and 10%MeOH aqueous solution. Then, the solvent composition in each standard solution must be prepared likewise as 1%DMSO and 10%MeOH aqueous solution.

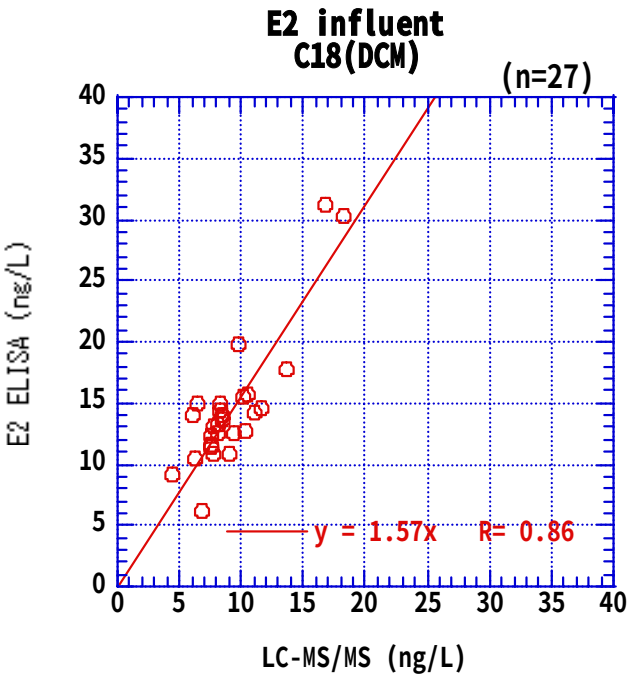
Flowchart: Method A

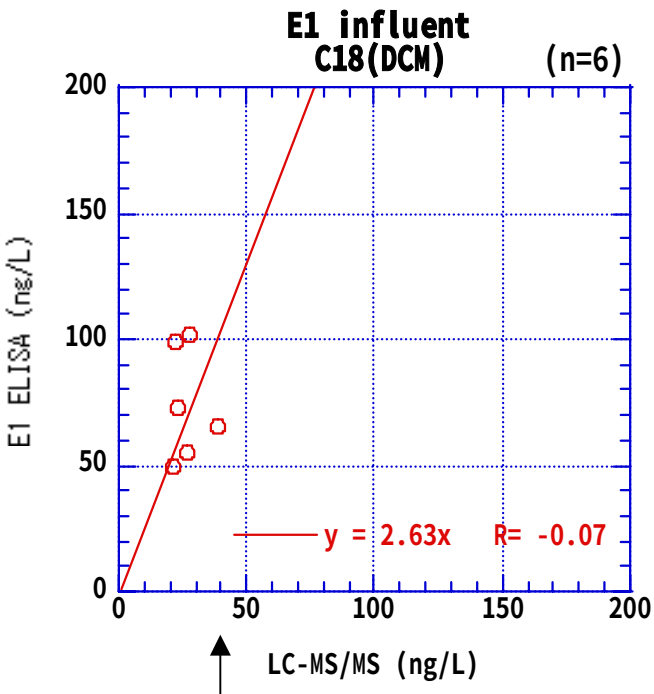


(2) Comparison between ELISA (Method A) and LC-MS/MS

Influent samples

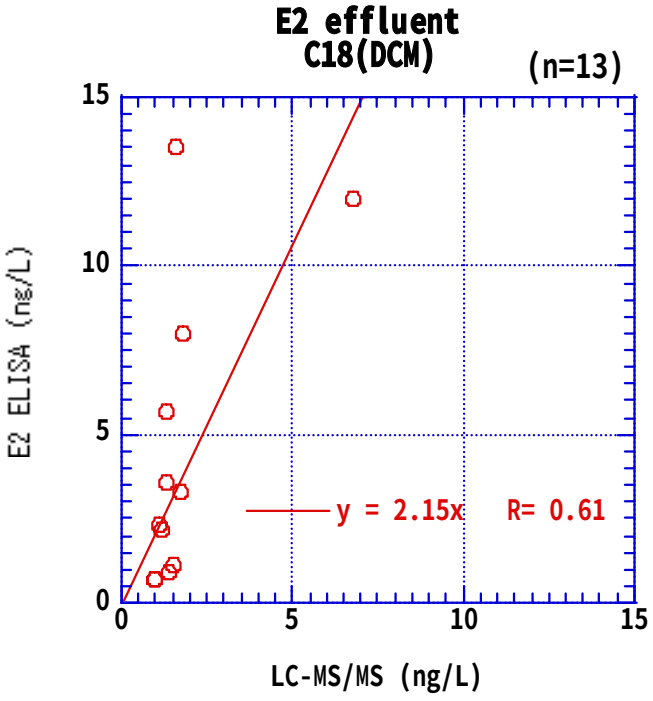
DCM: dichloromethane

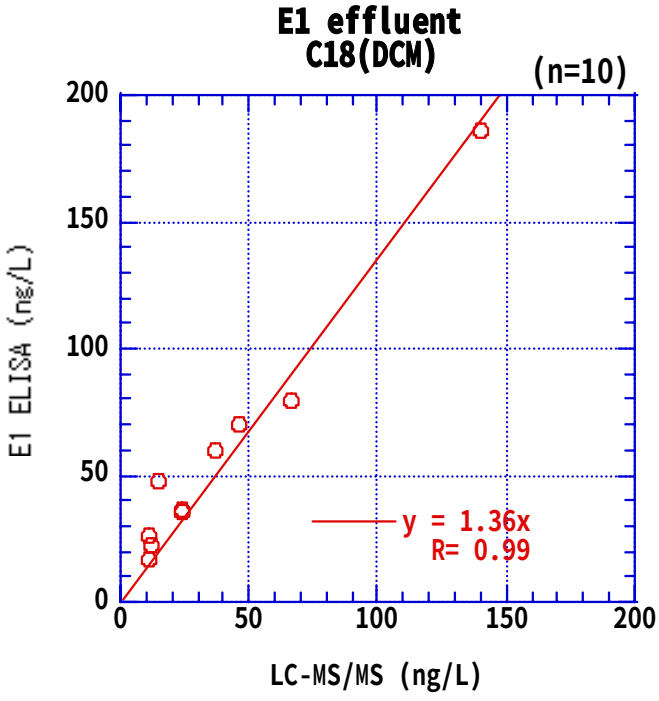




The values of E1 ELISA with Method A show a degree of overestimation, which will be alleviated with Method B.

Effluent samples





3. Solid Phase Extraction: Method B

(1) Protocol

1. Rinse a C18 cartridge with 5 ml of methanol and then 10 ml of distilled water (Preconditioning). To save time, suctioning with a vacuum pump is recommended. Flow rate, then, should not exceed 20ml/minute.
2. Then, pour the filtrate, prepared in Section 1 (Sample Filtration), through the C18 cartridge at a flow rate, no faster than 20ml/minute.
3. Wash the cartridge with 5ml of distilled water (up to 20ml/minute). Keep suctioning for about a minute to dry the cartridge. Then, wash the cartridge with 5ml of hexane (up to 20ml/minute).
4. Elute the analyte with 5ml of dichloromethane at a rate of 3ml/minute.
5. Evaporate the solvent with nitrogen gas.

   The typical setup is shown in the picture. Nitrogen gas, or (only if not available) compressed air, is supplied through pasteur pipettes into 10ml tubes containing eluted sample. Temperature is controlled at 40 - 50 degrees C with a water bath to accelerate evaporation.

6. Rinse an aminopropyl cartridge with 5ml of methanol beforehand as a preconditioning. Add 1ml of 100% methanol to the residue after evaporation and stir the mixture with a vortex. Then pour this methanol solution through an aminopropyl cartridge (3ml/minute). Keep the eluate of the loaded sample in a tube because E1 is unretained by an aminopropyl solid phase and, therefore, passes through. Whereas, matrix interferences like ionized E1 conjugates will be retained by the aminopropyl solid phase.
7. Elute the remaining estrone on the column with 5ml of methanol (3ml/minute) and receive the eluate into a tube, which contains the previously obtained eluate, ca. 1ml.
8. Evaporate the eluate, ca. 6ml, with nitrogen gas.
9. Add 100% methanol to the residue and stir the mixture with a vortex. Terminate the mixing and pour distilled water to adjust the content at 10% methanol (v/v).

If the initial amount of sample is 1liter, 1ml of the resulting 10% MeOH solution means 1000 fold concentration. Also prepare a 10-fold dilution from this concentrate so that the absorbance of either sample will fall in the dynamic range of the kit. Further dilutions may be necessary for some samples such as untreated wastewater.

Example:

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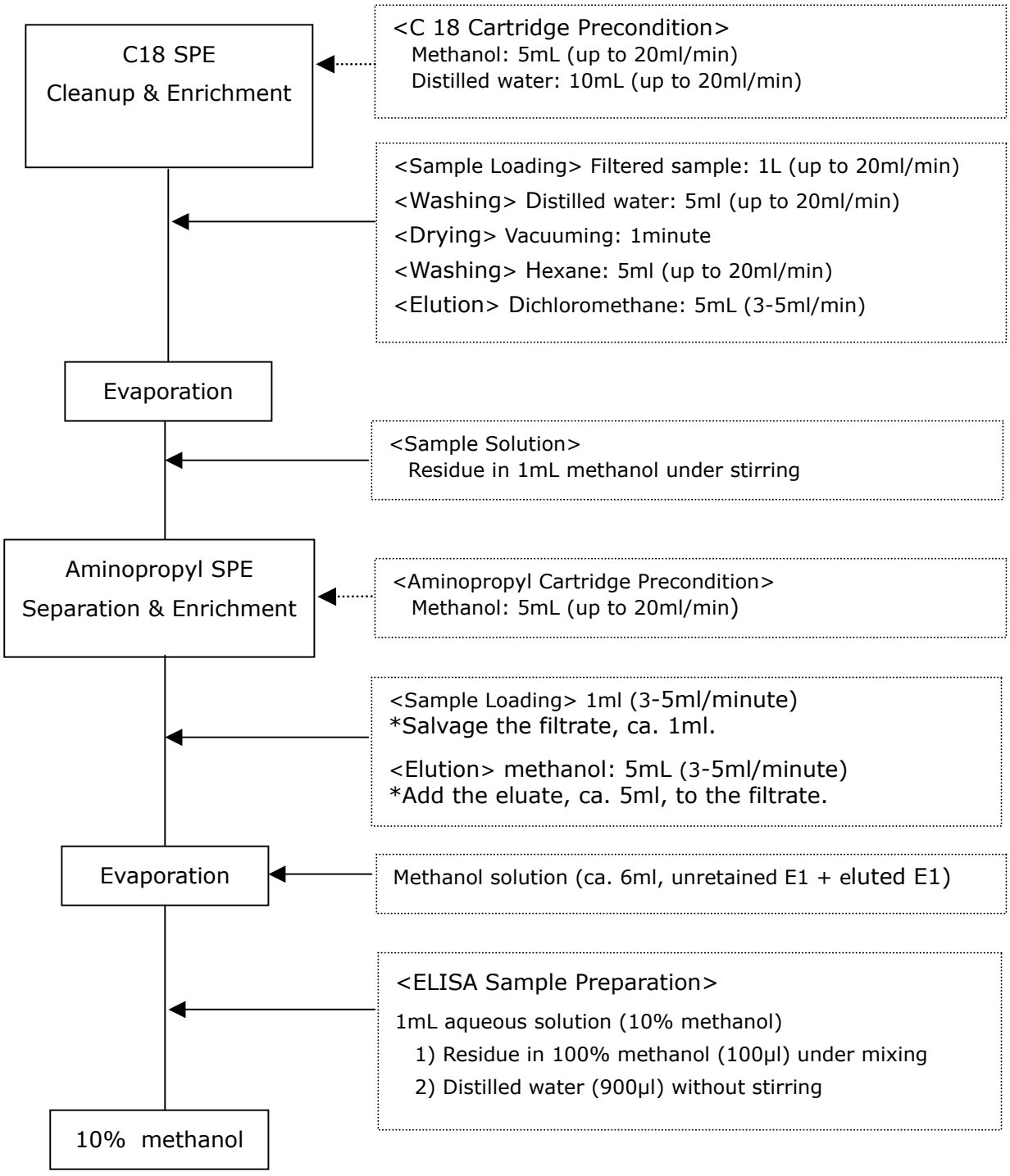
If most of the residue remains undissolved, evaporate the 10% methanol solution again with nitrogen gas. Add 10µL of 100% DMSO to the obtained residue and stir the mixture with a vortex. Then, add 100µL of 100% MeOH, 10 times of the volume of DMSO, terminate the mixing, and add distilled water until the total volume amounts to 1000µL (1ml). Then, the solvent composition in each standard solution must be prepared likewise as 1%DMSO and 10%MeOH aqueous solution.

Flowchart: Method B

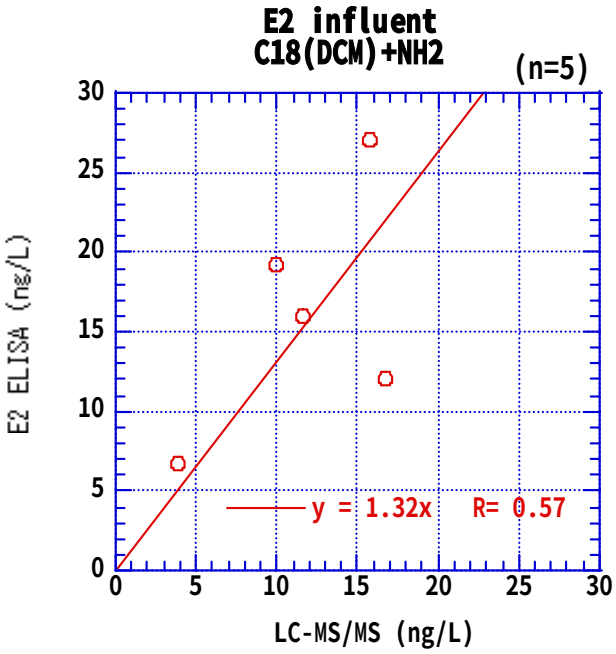


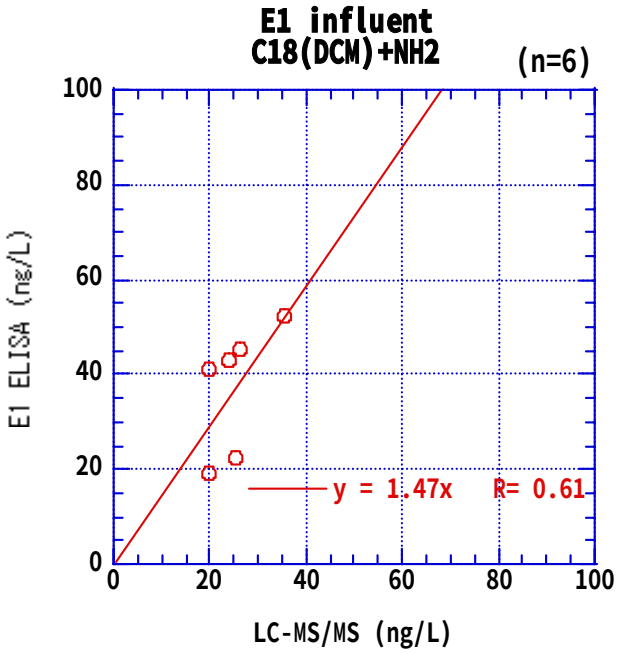
(2) Comparison between ELISA (Method B) and LC-MS/MS

Influent samples

DCM: dichloromethane

NH_2: aminopropyl solid phase





Effluent samples