CHAPTER III

EXPERIMENTAL

1. Apparatus

1.1. All analyses were performed on automated LC system (HP 1100 series from Agilent Technologies, USA.) consisted of auto-sampler, binary pump, on-line degaser, UV-Visible and fluorescence detector.

1.2. Milli-Q water system, model Millipore ZMQS5V00Y, Millipoore, USA.

1.3. Vortex Genie 2, Scientific Industries.

1.4. Rotary evaporator included cooling system (Julabo F33), heating bath, rota-vapor (Bűchi, Germany) and vacuum system (Bűchi, Germany).

1.5. HPLC column was ODS Hypersil C18, 5μm 250 x 4 mm i.d. (Agilent Technologies, USA) with guard column C18 (Merck, USA).

1.6. Mobile phase filter set included 300 mL glass reservoir, glass membrane holder, 1000 mL flask and metal clip, Millipore USA.

1.7. Water® vacuum pump, Millipore USA.

1.8. Balance, model Mettler AT 200, Mettler Toledo.

1.9. Autopipette and tips (Eppendorf, Germany)

1.10. Beakers 5, 10, 25, 50 and 100 mL.

1.11. Round bottom flasks 50, 100 and 250 mL.

1.12. Volumetric flasks 10 mL.

1.13. Glass centrifuge tubes 15 mL.

1.14. Round bottom tubes with screw caps 50 mL.

1.15. Round bottom flasks with PTFE screw caps 50 mL.

1.16. Glass cylinders 10, 25 and 100 mL.

1.17. 1.5 cm. i.d. glass column with PTFE stopcock and solvent reservoir.

1.18. TLC sheet F254 (silica gel 60, Merck USA).

1.19. Conical flasks 25 mL.

1.20. Glass test tubes 7 mL.

1.21. PTFE syringe filter, 0.45 μm, 13 mm i.d. (Agilent Technologies, USA).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

1.22. PTFE and Nylon membrane filter, 0.45 μm, 45 mm i.d. (Spectrum, USA.)

1.23. HPLC vials 2.0 mL with PTFE screw caps, Nation Science.

1.24. Nitrogen gas 99.99% purity, TIG, Thailand.

All glasswares for sample preparation were washed with detergent, rinsed with distilled water, dried, and rinsed with solvent before use.

2. Chemicals

2.1. Standard compounds.

Bisphenol-A-Diglycidyl Ether (**BADGE**), Bisphenol-F-Diglycidyl Ether (**BFDGE**), Bisphenol-A-bis (3-chloro-2-hydroxypropyl) (**BADGE.2HCl**), Bisphenol-F-bis (3-chloro-2-hydroxypropyl) (**BFDGE.2HCl**) were purchased from Fluka, Switzerland (Cas. No. 15138, 15144, 15136, and 15139 respectively). Low molecular weight BADGE and BFDGE were obtained from Ecopaint, Thailand. They had the consistency of yellow glue.

2.2. Organic solvents for BADGE synthesis.

Hexane, dichloromethane and ethylacetate were commercial grade and purified by distillation before use. Methyl *tert*-butyl ether (MTBE) and tetrahydrofuran (THF) were obtained from Merck, USA and Fluka, Switzerland.

2.3. HPLC mobile phase.

Methanol, isopropanol, and acetonitrile were HPLC grade and purchased from J.T. Baker, USA.

2.4. Organic solvents for extraction.

Acetonitrile and hexane (AR grade) were obtained from J.T. Baker, USA.

2.5. Reagent.

Hydrochloric acid, sodium bicarbonate and sodium chloride were analytical grade and obtained from Merck, USA.

2.6. Silica gel 230–400 mesh, Merck, USA.

2.7. Low molecular weight BADGE from Ecopaint, Thailand.

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

3. Synthesis of BADGE.HCl

Dissolved BADGE (3.40 g, 0.01 mol) in tetrahydrofuran (THF) and added 12 mL of 3% HCl (0.01 mol) into the solution. The mixture was autoclaved at 120 °C for 1 hour. The desired products were extracted twice with methyl *tert*-butyl ether (MTBE). The combined fractions were washed three times with 8% sodium bicarbonate and evaporated to dryness. The residue containing BADGE, BADGE.HCl and BFDGE.2HCl was separated on a silica gel (230-400 mesh) column using hexane/ethyl acetate (3:1 v/v) as eluant.

4. Preparation of the Standard Solutions

4.1 Preparation of the stock standard solutions 1000 ppm

Standard BADGE, BFDGE, BADGE.HCl, BADGE.2HCl and BFDGE.2HCl were weighed accurately to the nearest 0.1000 g and dissolved with acetonitrile in 10.00 mL volumetric flask respectively.

4.2 Preparation of 10 ppm working standard solutions

10 ppm standard solutions were prepared by mixing 15 µL of standard 1000 ppm solution into a 2.0 mL vial. Acetonitrile was added to make a total volume equaled 1500 µL.

4.3 Preparation of 10 ppm standard mixture

10 ppm standard mixture were prepared by mixing 15 µL of each standard 1000 ppm solution into a 2.0 mL vial. Acetonitrile was added to make a total volume equaled 1500 µL. The standard solutions were used for linearity study and to prepare calibration curves.

5. The Optimization of HPLC Separation

A HPLC equipped with a C18 column and water (A) and methanol (B) as mobile phase were used to develop optimum separation. The injection volume was 5 µL and the detector was fluorescence type. The optimization condition was determined by varying the mobile phase strength and flow rate. The separation of all

compounds was first tested with standard mixture and the condition was later tested with fish spiked matrix standard. Table 4-1 summarized this optimal HPLC condition.

6. The Specificity of All Standards

Individual standard solution (0.50 ppm) of BADGE, BADGE.HCl, BADGE.2HCl, BFDGE and BFDGE.2HCl were analyzed at this optimal HPLC conditions. The values of retention time, resolution and UV spectra of each standard was recorded and compared to the corresponded standard values.

7. The Study of Linearity

The procedure for linearity study of BADGE, BFDGE and their derivatives could be described as followed:

7.1. Series of 0.035, 0.050, 0.080, 0.100, 0.200, 0.400, 0.600, 0.800, 1.000 and 5.000 ppm standard solutions were prepared from 10 ppm standard mixture in step 4.3.

7.2. Solutions from 7.1 were analyzed twice by HPLC using condition in Table 4-1.

7.3. Peak area of standard solutions were recorded and regressed with corresponded concentrations to obtain linear regression lines with corresponded correlation coefficients (R^2).

ศูนย์วิทยทรัพยากร
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8. The Construction of Standard Calibration Curves

Standard calibration curves were constructed by:

8.1. Series of 0.035, 0.050, 0.080, 0.100, 0.200, 0.400, 0.600, 0.800 and 1.000 ppm standard mixture solution were prepared from 10 ppm standard mixture in step 4.3.

8.2. Series of standard solutions in step 8.1 were analyzed twice by HPLC using condition in Table 4-1.

8.3. Peak area of standards were recorded and regressed with corresponded concentrations to obtain regression lines and corresponded correlation coefficients (R^2).

9. The Determination of Limits of Detection (LoD)

The limit of detection (LoD) defined as the amount of analyte in standard solutions that yield a peak at signal-to-noise ratio equals to 3. The procedure to obtain LoD values can be described as:

9.1. Standard solutions concentration below 0.1 ppm was prepared by diluting 10 ppm of standard solutions as prepared in step 4.2.

9.2. Standard solutions from 9.1 were analyzed with HPLC at optimal condition listed in Table 4-1.

9.3. The signal-to-noise ratio of each standard was determined from corresponded peak with height at 3-times the baseline. LoD value is the corresponded concentration at this point.

10. The Determination of Limits of Quantitation (LoQ)

The limit of quantitation (LoQ) defined as the amount of analyte in standard solutions that yield a peak at signal-to-noise ratio equals to 10. The procedure to obtain LoQ values can be described as:

10.1. Standard solutions concentration below 0.1 ppm were prepared by diluting 10 ppm of standard in step 4.2.

10.2. Standard solutions in 10.1 were analyzed by HPLC at optimal condition listed in Table 4-1.

10.3. The signal-to-noise ratio of each standard was determined from corresponded peak with height at10-times the baseline. LoQ value is the corresponded concentration at this point.

11. The Extraction Procedure for Spiked Samples

There are 2 extraction procedures used in this work depend on type of samples.

11.1. Extraction procedure for fish in oil

Less than half the can lid was cut open. Oil phase was poured into the beaker until dried. Squeezed dried fish was transferred into separate beaker.

A. <u>Fish</u>

1) The squeeze-dried fish was homogenized by blender and transferred into a beaker.

2) Accurately weighted 5.00 g of homogeneous sample (fish) into 50 mL glass screw-top vial. Standard solutions were added into the sample.

3) Added 20 mL acetonitrile and vortexed for a few minutes.

4) Collected the acetonitrile fraction and repeated the extraction process once.

5) Combined acetonitrile fractions in 50 mL round bottom flask and added 10 g NaCl into the mixture.

6) Acetonitrile was evaporated close to dryness by a rotary evaporator at 35 °C. The residue was transferred into 15 mL centrifuge tube.

7) The solution was dried under a steam of nitrogen. The residue was dissolved with 1.00 mL acetonitrile.

8) The solution was extracted with 1.00 mL hexane by vortexing for a few minutes. The hexane layer was removed and repeated the hexane extraction was repeated one more time.

9) The acetonitrile layer was analyzed by HPLC.

B. <u>Oil phase</u>

1) Accurately weighted 5.00 g of oil into 50 mL glass screw-top vial. Spiked the oil with standard solutions.

2) The sample from step 1) was extracted with 20 mL acetonitrile twice. Combined the acetonitrile fractions in a round bottom flask and evaporated the solvent close to dryness.

3) Transferred the solution in step 2) into 15 mL glass centrifuge tube and dried the solution under a steam of nitrogen.

4) Dissolved the residue with 1.00 mL acetonitrile and extracted with 1.00 mL hexane twice.

5) The remaining acetonitrile layer was subjected for HPLC analysis.

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

11.2. Extraction procedure for fried food samples

1) Less than half of the top lid was cut open. Remove the food content into a blender. Homogenized the food until uniform and transferred into a beaker.

2) Accurately weighted 5.00 g of homogeneous sample into 50 mL glass screw-top vial. Standard solutions were spiked into the sample.

3) Added 20 mL acetonitrile and vortexing for a few minutes.

4) Collected the acetonitrile fraction and repeated step 3) once.

5) Combined the acetonitrile fractions in a 50 mL round bottom flask and added 10 g NaCl into the mixture.

6) Evaporated the solvent close to dryness by a rotary evaporator. The residue was transferred into a 15 mL glass centrifuge tube.

7) The remaining solvent was dried under a steam of nitrogen. The residue was dissolved with 1.00 mL acetonitrile.

8) The solution was extracted with 1.00 mL hexane by vortexing for a few minutes. Removed the hexane layer and repeated the extraction once.

9) The acetonitrile layer was subjected for HPLC analysis.

12. The Study of Precision and Percent Recovery

Precision and percent recovery of each standard was used for indicating the uncertainty of the method. Percent recovery specifies method accuracy. In this study, we spiked the standard solutions into blank (clean tuna), extracted, and determined the concentration of each standard recovered by the developed extraction procedure. The spiking levels of standard solutions were at LoQ and at 5-fold LoQ. The procedure for precision study could be described as followed:

12.1. Blank tuna (clean tuna) was homogenized by blender and transferred into a beaker.

12.2. Accurately weighted 50.00 g of homogenized blank tuna into a glass screw cap vial and spiked with standard solutions.

12.3. Spiked blank was mixed to uniformity and divided into 10 equal portions.

12.4. Each portion was extracted with 20 mL acetonitrile as described in section 11 step A3-A9, followed by HPLC analysis.

12.5. Peak area and corresponded concentration data were regressed to obtain a regression line that used to back calculate the recovered spiked concentration. Results are reported as standard deviation, coefficient of variance, and percent recovery.

12.6. Acceptable recovery procedure must recover more than 60% of spiked concentration with coefficient of variance less than 20%.

13. The Study of Method Robustness

Robustness was the capacity of a method to remain unaffected by small deliberate variations in method parameters. Two parameters of the extraction procedure were changed to study method robustness. The procedure for robustness study could be described as followed:

13.1. Variation of acetonitrile volume for extraction

The volume of acetonitrile for extraction was deliberately changed from 20 mL to 30 mL. The extraction procedure was similar to Section 11.

1) Accurately weighted 30.00 g of blank tuna sample into screw cap flask and spiked with standard solutions.

2) Mixture from step 1) was divided into 6 equal portions. Each portion was extracted with 30 mL acetonitrile (instead of 20 mL) using procedure described in 11.

3) Acetonitrile fractions were collected and evaporated as per Section 11 step A3-A9. The solution was subjected to HPLC analysis.

4) Peak area and corresponded concentration data were recorded and used to back calculate the recovered spiked concentrations. Student t-test was used to compare the significant of the means obtained from both procedures.

ศูนย์วิทยทรัพยากร
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13.2. Variation of evaporating temperature:

The heating temperature of the rotary evaporator was changed from 30 °C to 40 °C. The procedure for this study could be described as followed:

1) Accurately weighted 30.00 g of blank tuna sample into screw cap bottom and spiked with standard solutions.

1) Accurately weighted 30.00 g of blank tuna sample into screw cap bottom and spiked with standard solutions.

2) The mixture from 1) was mixed well and divided into 6 portions. Extracted each portion with 20 mL acetonitrile as described in Section 11.

3) Once reached step A6 in Section 11, set the heating temperature of the rotary evaporator step to 40 °C and evaporated the solvent close to dryness.

4) Followed step A7 to A9 in Section 11 until sample was ready for HPLC analysis.

5) Peak area and corresponded concentration data were recorded and used to back calculate the recovered spiked concentrations. Student t-test was used to compare the significant of the means obtained from both procedures.

14. Real Sample Preparation

Twenty samples of oil-based canned food were chosen form Thai market. The analyses were done on 2 parts: foodstuff and empty cans.

14.1. Foodstuff

Half of the lid was cut open and the food content was transferred into a beaker. For tuna in oil, both oil and fish were separated and each part was analyzed separately. Oil was diluted to suitable concentration before analyzed by HPLC. The fish (squeezed out oil) was homogenized and extracted with acetonitrile as described in Section 11. Extraction was repeated 3-times per can.

14.2. Empty can.

After foodstuff was removed, the empty can was cleaned with household detergent and air dried. Hexane was used to rinse off fat residues from coatings and the can was air dried before Beilstien's Test.

<u>The Beilstein's Test</u>

The Beilstein's test (flame test) was used to check types of can coating by detecting chlorine in polyvinylchloride (PVC). The procedure was described as followed:

14.2.2. Clean the copper wire surface from existing chlorine residue by scorching in hot flame.

14.2.2 Applied small amount of scrapped polymer on the clean wire surface.

14.2.3. Insert the wire into flame. For positive result (organosol presented), the green flame was observed. Normal orange flame indicated negative result (other polymer coating).

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