CHAPTER III

EXPERIMENTAL

3.1 Instrument and Apparatus

3.1.1 High Performance Liquid Chromatography (HPLC): A module 1100™ consists of automatic degasser, binary pump, autosampler, column thermostat, diode-array detector, and fluorescence detector, and mass selective detector with electrospray ionization interface. Agilent Technologies, USA.

3.1.2 Milli-Q, Ultrapure Water Systems, with Millipak® 40 Filter Unit 0.22 µm, model Millipore ZMQS5V00Y, Millipore, USA.

3.1.3 Vortex mixer, Scientific Industries.

3.1.4 A rotary evaporator consists of cooling (Julabo F33), Büchi Heating Bath B-490, Büchi Rotavapor R-200, Büchi Vac® V-500.

3.1.5 HPLC column: Hypersil ODS, C18 Column 250 x 4.0 mm I.D., 5 µm, Agilent Technologies, USA.

3.1.6 A Guard Cartridge Holder, Agilent Technologies, USA with C18 High Performance guard column, E. Merck, Darmstadt, Germany.

3.1.7 A Water® Vacuum Pump, model DOA-V130-BN, with Pressure Regulator, Millipore, USA.

3.1.8 A Glass Filter Holder Set (300 mL Funnel, 1 L Flask, Glass base and tube cap, and 47 mm Spring Clamp) for HPLC mobile phase filtration, Millipore, USA.

3.1.9 Volumetric flasks 10.00 and 25.00 mL.

3.1.10 Beakers 10, 50, 150, 250, 600, and 1000 mL.

3.1.11 Round bottom flasks 50 and 100 mL.

3.1.12 Conical-bottom glass centrifuge tubes 15 mL.

3.1.13 Round-bottom glass centrifuge tubes 50 mL.

3.1.14 Graduated cylinders 10.0, 50.0 and 100.0 mL.

3.1.15 Volumetric Pipettes 10.00 mL.

3.1.16 Micro-pipettes and tips.

3.1.17 Glass column with PTFE stopcock.

3.1.18 TLC aluminum sheets, silica gel 60 F254, E. Merck Darmstadt, Germany.

3.1.19 Filter Membrane 47 mm, 0.45 μm, type Teflon and Nylon, Agilent Technologies, USA.

3.1.20 Syringe filter, Nylon 13 mm, 0.45 μm, Agilent Technologies, USA.

3.1.21 HPLC vials 2 mL with caps, Agilent Technologies, USA.

3.1.22 N_2 Gas 99.99% purity, TIG, Thailand.

All glass apparatus was washed thoroughly in detergent, rinsed with double distilled water and then rinsed with elution solvent before used.

3.2 Chemicals

3.2.1 The Standard Compounds

Bisphenol-A-diglycidyl ether (**BADGE**), Bisphenol-A-(3-chloro-2-hydroxypropyl) glycidyl ether (**BADGE.HCl**), Bisphenol-A-bis(3-chloro-2-hydroxypropyl) ether (**BADGE.2HCl**), Bisphenol-A-(2,3-dihydroxypropyl) glycidyl ether (**BADGE.H_2O**), Bisphenol-A-bis(2,3-dihydroxypropyl) ether (**BADGE.2H_2O**), Bisphenol-A-(3-chloro-2-hydroxypropyl) (2,3-dihydroxypropyl) ether (**BADGE.HCl.H_2O**), Bisphenol-F-diglycidyl ether (**BFDGE**), Bisphenol-F-bis(3-chloro-2-hydroxypropyl) ether (**BFDGE.2HCl**), Bisphenol-F-bis(2,3-dihydroxypropyl) ether (**BFDGE.2H_2O**) which had percent purity of 97, 95, 99, 97, 97, 98, 97, 95, and 97%, respectively were supplied by Fluka Chemika, Switzerland.

3.2.2 Organic Solvents

Hexane, ethyl acetate (J. T. Baker Chemical Company, Deventer, Holland), tetrahydrofuran (E. Merck, Darmstadt, Germany), and *tert*-butyl methyl ether (Fluka Chemika, Switzerland) were analytical grade (AR Grade). Methanol and acetonitrile were HPLC grade purchased from J. T. Baker Chemical Company, Deventer, Holland.

3.2.3 Reagents

Hydrochloric acid, acetic acid, di-sodium hydrogen phosphate dihydrate, and sodium chloride were analytical grade supplied by E. Merck, Darmstadt, Germany. Anhydrous Sodium sulfate was purchased from J. T. Baker Chemical Company, Deventer, Holland.

3.2.4 Silica gel 230-400 mesh, E. Merck, Darmstadt, Germany

3.2.5 Bisphenol-F-diglycidyl ether for synthesis (Thai Epoxy CO., LTD.)

3.3 Preparation methods of BFDGE.H_2O, BFDGE.HCl, and BFDGE.HCl.H_2O

The non-symmetric derivatives bisphenol-F-glycidyl-(2,3-dihydroxypropyl) ether (**BFDGE.H_2O**), bisphenol-F-glycidyl-(3-chloro-2-hydroxypropyl) ether (**BFDGE.HCl**) and bisphenol-F-(3-chloro-2-hydroxypropyl)-(2,3-dihydroxypropyl) ether (**BFDGE.HCl.H_2O**) were not commercially available. They were prepared and purified in-house for use.

BFDGE.H_2O

BFDGE (6.24 g, 0.02 mol) was dissolved in tetrahydrofuran (THF) and added to 20 mL of 3% acetic acid (0.01 mol). The mixture was stored for 3 days at ambient temperature (32 °C). The products were extracted twice with 15 mL *tert*-butyl methyl ether (MTBE). The combined extracts were washed three times with 10 mL each of 8% sodium bicarbonate and evaporated to dryness. The residue containing BFDGE, BFDGE.H_2O and BFDGE.2H_2O was separated on a silica gel (230-400 mesh) column using hexane/ethyl acetate (1:6 v/v) as eluant.

BFDGE.HCl

BFDGE (3.12 g, 0.01 mol) was dissolved in THF and added to 10 mL of 1.5% v/v HCl in THF (0.005 mol). After 2 minutes, the products were extracted and washed as in previous procedure. The extract containing BFDGE, BFDGE.HCl, and

BFDGE.2HCl were separated on a silica gel column using hexane/ethyl acetate (4:1 v/v) as eluant. The fractions containing BFDGE.HCl were combined and evaporated to dryness.

BFDGE.HCl.H_2O

BFDGE (6.24 g, 0.02 mol) was dissolved in THF and added to 20 mL of 1.5% v/v HCl (0.01 mol). The mixture was stored for 3 days at ambient temperature. The products were extracted and washed as explained previously. After evaporation to dryness, purification was carried out on a silica gel column in order to remove the BFDGE, BFDGE.H_2O, BFDGE.$2H_2O$, BFDGE.HCl and BFDGE.2HCl using hexane/ethyl acetate (1:4 v/v) as eluant.

The fractions containing pure products were combined and tested for their chromatographic properties by HPLC and fluorescence detection, excitation and emission wavelengths of 227 and 313 nm, respectively; and UV detection at 227 nm. Characterization was further performed by LC-ESI-MS under flow injection analysis mode (FIA). An isocratice elution was carried on a mixture (50:50, v/v) of (**A**) 95%water-acetonitrile (10 mM ammonium acetate) and (**B**) 5%water-acetonitrile (10 mM ammonium acetate) at flow rate 0.50 mL/min. The working conditions for positive-ion mode electrospray ionization were as follows: the drying nitrogen gas temperature was set at 350 °C; flow rate at the capillary region equaled 10 L min^{-1}; the capillary was held at a constant potential of 3,500 V relative to the potential of counter electrode. The fragmentor voltage was varied to obtain optimum fragmentation.

3.4 Preparation of Standard Solutions

3.4.1 Stock Standard Solutions in Acetonitrile

The 1,000 ppm stock standard solutions of each compound were prepared by dissolving 0.0100 g of each standard and diluting them to the mark with acetonitrile in 10.00 mL volumetric flasks.

3.4.2 Stock Synthesis Solutions

The synthesis solutions were prepared by dissolving all of the obtained paste (0.0623, 0.2057, and 0.1652 g for BFDGE.H_2O, BFDGE.HCl, and BFDGE.HCl.H_2O respectively) with acetonitrile in 25.00 mL volumetric flasks.

The 1000 ppm synthesis solutions of each compound were prepared by pipetting 602, 182, and 227 μL of BFDGE.H_2O, BFDGE.HCl, and BFDGE.HCl.H_2O respectively into each stock solution contained in a 2 mL vial and diluting to 1,500 μL with acetonitrile.

3.4.3 Mixture Solution

The 10.0 ppm of standard mixture solutions were prepared by pipetting 10.0 μL of each 1,000 ppm stock solution into 2 mL vial and then diluting to 1,000 μL with methanol by autopipette. The standard solutions for preparation of the calibration curves were prepared from these solutions.

3.5 The Study of High Performance Liquid Chromatographic Conditions

The HPLC condition was developed by varying the flow rate and percentage of mobile phase in order to obtain the optimum HPLC conditions. 5 μL of the standard solutions, spiked standard solutions and sample solutions were injected into the HPLC under optimum HPLC condition listed in Table 4.3. A chromatogram of standard mixture solution was shown in Figure 4.5.

3.6 The Study of Selectivity Evaluation of the HPLC Condition

The selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other component in the sample. In this study, the selectivity of HPLC was determined by the peak retention time, resolution, and UV spectra at 227 nm of each analyte at the optimum chromatographic condition in Table 4.3. The results are shown in Table 4.4.

3.7 The Study of Standard Calibration Curves

The procedure to study the calibration curves of standard BADGE, BFDGE and their derivatives can be described as followed:

3.7.1 The concentrations of standard solutions: 0.0160, 0.100, 0.200, 0.400, 0.600, 0.800, 1.00 ppm were injected respectively into HPLC under the optimum condition (Table 4.3).

3.7.2 The relationships between concentration and peak area were plotted. The intercepts, slopes, and correlation coefficients from each curve are summarized in Table 4.5.

3.8 The Study of Linearity Range

The procedure for studying the linearity ranges of standard BADGE, BFDGE and their derivatives can be described as followed:

3.8.1 The concentrations of standard solutions: 0.0160, 0.100, 0.200, 0.400, 0.600, 0.800, 1.00, 3.00, 5.00, 7.00, and 10.0 ppm were injected respectively into HPLC under the optimum condition (Table 4.3).

3.8.2 The relationships between concentration and peak area were plotted. The intercept, slope, and correlation coefficient of all compounds are summarized in Table 4.6.

3.9 The Study of Detection Limits and Quantitation Limits

The detection limit and quantitation limit of the instrument were defined as the amount of analytes in standard solution that yield as peaks at signal-to-noise ratio equal to 3 and 10, respectively. The procedure can be described as followed:

3.9.1 The 10.0 ppm standard mixture solution was prepared from respective 1,000 ppm stock solution.

3.9.2 The 1 ppm standard mixture solutions and of concentration below 1 ppm were prepared by diluting 10.0 ppm of standard mixture solution from step 3.9.1 with methanol.

3.9.3 The standard mixture solution from step 3.9.2 was injected into HPLC system under the optimum condition (Table 4.3). The peak signals of each compound were measured from the chromatograms.

3.9.4 The detection limit and quantitation limit of each compound were determined from the concentration that gave the peak signal as high as 3 and 10 times of the baseline signal, respectively.

3.9.5 The detection limit and quantitation limit of each compound are illustrated in Table 4.7.

3.10 The Procedures for Extraction of Oil-in-Water-Based Samples

The extraction procedure of spiked standard solution can be described as followed:

3.10.1 A portion of 10.00 g of homogenized food sample was extracted with 15.00 mL of *tert*-butyl methyl ether.

3.10.2 10.00 mL of the extracted solution was transferred into a 50 mL round bottom flask and extracted again with 15.00 mL of *tert*-butyl methyl ether.

3.10.3 The new fraction (10.00 mL) was combined with the fraction from step 3.10.2 and removed the solvent on a rotary evaporator to dryness at 34 °C.

3.10.4 The residue was extracted three times with 5.00 mL methanol.

3.10.5 The methanol fractions were combined in a 50 mL round bottomed flask and the solvent was removed on a rotary evaporator to dryness at 34 °C.

3.10.6 The dried residue was re-dissolved in 1.00 mL methanol and then filtered through 0.45 μm filter for HPLC analysis.

3.11 The Effect of Acidity on Aqueous-Based Samples

The effect of acidity on aqueous-based samples was studied in lychee in syrup matrix by comparing sample blank, spiked sample, sample bank (adjusted pH), and

spiked sample (adjusted pH). Sample preparation procedure can be described as followed:

3.11.1 Sample blank was prepared by extracting 10.00 g of homogenized lychee with 15.00 mL acetonitrile and 10 g sodium chloride.

3.11.2 10.00 mL of the extracted solution was transferred into a 50 mL round-bottom glass tube.

3.11.3 After extracted as per step 3.11.2, the sample was extracted again with another 15.00 ml of acetonitrile. The new fraction was combined with the fraction from step 3.11.2 and 10 g of sodium sulfate was added to the solution.

3.11.4 The solvent was removed by a rotary evaporator at 34 °C.

3.11.5 The dried residue was re-dissolved in 1.00 mL methanol and filtered through 0.45 μm filter for HPLC analysis.

3.11.6 Spiked samples were prepared by adding standard mixture solution to 10.00 g homogenized sample making final concentration of standard equal 10-fold MQL.

3.11.7 Each spiked sample in step 3.11.6 was extracted by procedure in step 3.11.1 to 3.11.5.

3.11.8 For sample blank (adjusted pH) and spiked sample (adjusted pH), the homogenized samples were adjusted to pH 7.0 by 1.0 M di-sodium hydrogen phosphate dehydrate before extracted following step 3.11.1 to 3.11.5.

3.12 The Study of Matrix Calibration Curve

The matrix calibration curves were studied in fish matrices. The procedure for the study can be described as followed:

3.12.1 Blank solution was prepared by the extraction procedure according to the procedure in 3.10.

3.12.2 Standard mixture solutions at 0.0160, 0.100, 0.200, 0.400, 0.600, 0.800, and 1.00 ppm were prepared using the blank solution in 3.12.1 as diluting solvent instead of methanol.

3.12.3 Standard mixture solutions in fish matrix from 3.12.2 were injected respectively into HPLC under the optimum condition (Table 4.3).

3.12.4 The matrix calibration curves were constructed by plotting concentration versus peak area. Calibration parameters of all compounds are summarized in Table 4.8.

3.13 The Study of Matrix Effect

The matrix effect in sample was studied using fish matrix as a representative of all samples. The statistical analysis of the calibration curve of standard mixture in methanol solution (section 3.7) was performed against the matrix solution curve (section 3.12) using two tailed paired *t*-test at 95% confidence level. The *t*-value is reported in Table 4.9.

3.14 The Study of Method Quantitation Limits (MQL)

The method quantitation limit was defined as the amount of analyte in spiked standard solution that yields a peak at signal-to-noise ratio equal to 10. The procedure can be described as follows:

3.14.1 The spiked samples were prepared by spiking each volume of standard solution into 10.00 g of homogenized fish and extracted by procedure in Section 3.10

3.14.2 Blank sample was prepared in the same way as spiked samples but not to be added to the standard solution.

3.14.3 The blank and spiked sample from step 3.14.1 and 3.14.2 were injected into HPLC system under the optimum condition (Table 4.3). Peaks of each compound were measured from the chromatograms.

3.14.4 The MQL of each compound was found from the concentration that gave the peak signal as high as 10 times of the baseline signal. The result is described in Table 4.10.

3.15 The Study of Method Detection Limits (MDL)

The method detection limit is considered the lowest concentration that can be detected after samples have gone through the entire sample preparation scheme prior to analysis. In this research, the method detection limit were obtained by calculation from the method quantitation limit by using the signal-to-noise equal to 3 in Section 3.14 .

3.16 The Study of Method Precision

Precision is the amount of scatter in the results obtained from multiple analyses of homogeneous sample(s). In this study, precision was divided into intra-assay precision or repeatability obtained by repeatedly analyzing the same sample in one day and intermediate precision which was obtained when the assay was performed on different days. The study was performed by extraction of spiked standard solutions at Method Quantification Limits (MQL) and 5-fold MQL concentration levels, as shown in Table 4.11. The extractions at each level was repeated 6 times within each batch for an intra-assay precision study. Then, the extraction at each level was repeated on 3 different days for an intermediate precision study.

The procedure for the study of method precision can be described as follows:

3.16.1 *For oil-in-water-based sample:*

1) Six spiked samples were prepared by pipetting standard mixture solution at MQL level into the 10.00 g each of homogenized fish.
2) Each spiked sample in step 1) was extracted by procedure in Section 3.10
3) The final concentration of each compound was calculated by using linear equation and showed the recovery as percentage of the original spiked concentration.
4) The intra-assay precision of this method was calculated and reported in form of percent relative standard deviation (%RSD) of each compound. The percent recoveries are shown in Table 4.12-4.14.

5) For the intermediate precision, the extraction at this level was repeated on another 2 different days. The summary of the results of 3 different days is illustrated in Table 4.15.
6) For the higher level concentration (5-fold MQL), spiked samples were prepared by pipetting standard mixture solution into 10.00 g each of homogenized fish.
7) The extraction and calculation were similar to the step 2) to 5). The results are described in Table 4.16-4.19.

3.16.2 *For aqueous-based sample:*

1) Six spiked samples were prepared by pipetting standard mixture solution at 5-fold MQL into the 10.00 g each of homogenized lychee in syrup.
2) Each spiked sample in step 1) was extracted by the procedure in Section 3.11
3) The extraction was repeated on 2 different days.
4) The calculation was similar to the previous section (3.16.1) and the results summarized in Table 4.20-4.23.

3.17 The Study of Method Accuracy

The accuracy of a method is the closeness of the measured values to the true value (concentration) of the sample. There are several ways for determining of the method accuracy such as using the method to analyze a Certified Reference Material, comparing results from two or more different analytical methods, analyzing a blank sample spiked with known addition of analyte, etc. In this research, spiking was chosen to determine the accuracy because the Certified Reference Material and the second analytical method were unavailable at time of study.

The accuracy was performed by spiking analytes into fish matrices at two level concentrations for the method of oil-in-water-based sample and extracted by the procedure in Section 3.10 on three occasions. For the method of aqueous-based sample, the accuracy was obtained by spiking analytes in lychee in syrup matrices and extracted by the procedure in Section 3.11 on three different occasions. The accuracy

of method was determined by the mean recovery for each compound and the results are described in Table 4.24.

3.18 Sample Stability

The stability of the analytes was determined by using fish matrix spiked with 12 compounds followed by normal extraction procedure in Section 3.10. These extracted solutions were divided into 8 vials, stored under the same conditions, and then analyzed on different days within a 42-days period. The concentrations were back calculated using linear equations of freshly prepared standard solutions and the recoveries were reported as percentage of the original spiked concentration. The recovery results are summarized in Table 4.25 and illustrated as control charts in Figure 4.14-4.25.

3.19 The Study of Method Robustness

Robustness is the capacity of a method to remain unaffected by small variations in method parameters. It is a measurement of the reliability of a method. Method robustness can be evaluated by varying certain method parameters throughout a known range and determine the effect as percent recoveries of spiked standard solution. In this study, the effect of 2 parameters (evaporation temperature and number of extraction with *tert*-butyl methyl ether) were studied by comparing percent recovery values with the normal procedure (Section 3.10). The procedure for the study of the robustness can be described as followed:

3.19.1 Six spiked samples were prepared by pipetting standard mixture solution at 5-fold MQL into the 10.00 g each of homogenized fish.

3.19.2 Each spiked sample in step 3.19.1 was extracted by procedure in 3.10

3.19.3 The extraction was repeated as step 3.19.2 but changed the following parameters:

1) Rotary evaporator was set at 40 °C.
2) Extract with *tert*-butyl methyl ether 1 times (30 mL).

3.19.4 The final concentrations of each compound were back calculated using linear regression. Percent recoveries of the original spiked concentration are summarized in Table 4.27.

3.19.5 The data were compared with normal procedure by two tailed paired *t*-test at 95% confidence level. The *t*-values are reported are reported in Table 4.28.

3.20 Determination of BADGE, BFDGE and Their Derivatives in Canned Foods

Two cans each of various canned food samples were randomly selected from supermarkets in Bangkok metropolitan area. The analyses were divided into 4 parts:

3.20.1 Analysis of oil-in-water based foodstuff

Fifteen types of canned foods were chosen:

1. Sardines in tomato sauce
2. Green curry fried sardines
3. Tom Yum sardines
4. Pork green curry
5. Tuna steak
6. Tuna with ginger
7. Seasoned vegetarian bamboo shoot with mushroom
8. Chuchee sardines
9. Vegetarian Palo soup
10. Seasoned vegetarian cabbage with mushroom
11. Chicken red thick curry
12. Namprik tuna
13. Beef Masman curry
14. Tuna steak in brine
15. Tuna with chilli basil leaf

The procedure for determining of BADGE, BFDGE, and their derivatives in each canned food sample can be described as followed:

1) The whole content of each can was homogenized and a portion of 10.00 g of the homogenized sample was extracted according to the procedure 3.10
2) The peaks obtained from the chromatogram were matched against the retention time and the UV spectra of standard compounds.
3) The concentration of each compound was calculated using the external standard calibration method. The results are summarized in Table 4.29

3.20.2 Analysis of aqueous-based foodstuff

Ten types of canned foods were chosen:

1. Orange juice
2. Tomato juice
3. Coffee
4. Carbonated beverages
5. Lychee in syrup
6. Mustard green leaf
7. Rumbutan in heavy syrup
8. Pineapple pieces in heavy syrup
9. Young sweet corn in brine
10. Champignon mushrooms in brine

The quantitation procedure was similar to that in Section 3.20.1 while the whole content of each can was homogenized and a portion of 10.00 g of the homogenized sample was extracted according to the procedure in 3.11. The results are illustrated in Table 4.32.

3.20.3 Analysis of empty cans

1) Lids were opened less than half and rinsed with soapy water. After drying, rinsed with hexane to eliminate fat or oil.
2) Filled can with acetonitrile to known proportion of 50% internal volume, so that the solvent was in contact with half of the surface of all parts.
3) Extraction was performed for 24 hours at room temperature.
4) The extract was injected into HPLC and calculated the concentration by using the external standard calibration method. The results are described in Table 4.30 and 4.33.

3.20.4 Beilstein test

Beilstein test was used to check the presence of chlorine in the coating. Some lacquer was scraped from the metal surface. A copper wire was heated above the blue flame and then the lacquer was picked up and burn on the flame. A green color flame showed that the coating had chlorine or "Beilstein-positive" as shown in the APPENDIX C compared with the Beilstein-negative. The Beilstein test results are shown in Table 4.31 and 4.34.