CHAPTER THREE

EXPERIMENTAL

3.1. Apparatus and instruments

3.1.1. Spectrofluorometry

Fluorescence excitation and emission spectra were recorded with a Perkin Elmer Model LS 50B spectrofluorometer (PerkinElmer Corp., Norwalk, CT) using the FL Winlab software (PerkinElmer). The slit-width was set at 3.0 nm with a scanning speed of 1000 nm/min ranging from 200 to 600 nm.

3.1.2. GC

3.1.2.1. Mass spectrometry (MS)

The system used was a Hewlett-Packard (Palo Alto, CA) HP-5890 GC equipped with a HP-5970 MS detector, and fitted with a split/splitless injection port. The column used was a HP-5 MS cross-linked with 5 % phenyl methylsiloxane capillary column of 30 m x 0.32 mm i.d. The carrier gas employed was helium set at a flow rate of 0.80 mL/min. Data was acquired with the HP-59970 MS ChemStation.
3.1.2.2. Flame ionization detection (FID)

An Agilent 6890 (Agilent Technologies Inc, Wilmington, DE) GC equipped with an Agilent 7683 auto-injector was operated on the splitless mode. The separation was performed on a capillary column (HP-5 cross-linked with 5% phenyl methylsiloxane, 30 m x 0.32 mm id., Hewlett-Packard, Palo Alto, CA) with helium as the carrier gas set at 0.8 mL/min.

3.1.3. HPLC

The system consists of a pump (Jasco PU-980; Jasco International Co., Ltd, Tokyo, Japan), a 3-inline degasser (Jasco DG-980-50), a ternary gradient unit (LG-980-02S), an autosampler (Jasco 851-AS), and a column oven (Jasco CO-965).

The detectors were a programmable fluorescence detector (Jasco FP-970), a diode array detector (Jasco MD-1510), UV-visible detector (UV-975) and an SEDEX 55 ELSD (SEDERE, Alfortville, France). All the data was acquired using a Borwin 1.21 (JMBS Developpements, Le Fontanil, France) chromatographic software.
3.2. Quantitative analysis

3.2.1. Eutectic mixtures of diphenyl oxide and biphenyl

3.2.1.1. Reference standards

Diphenyl oxide and biphenyl, 98% purity, were purchased from Fluka Chemika AG (Buchs, Switzerland). 10 mg of diphenyl oxide standard was accurately weighed and dissolved in methanol. The solution was transferred to a 100-mL volumetric flask and diluted to the mark with methanol. 1 mL of the stock solution was then pipetted into a 10-mL volumetric flask which was further diluted and mixed. The solutions for biphenyl were prepared in a similar manner.

3.2.1.2. Working standards

Dowtherm $A^{\text{TM}}$ was supplied by Dow Chemical Company (Midland, MI). A 100-μg/mL stock solution was prepared by dissolving 10 mg Dowtherm $A^{\text{TM}}$ with methanol to the mark in a 100-mL volumetric flask. The appropriate aliquots of the stock solution in methanol were diluted to produce working standards with 10, 5, 1, 0.1, 0.01, and 0.005 μg/mL. Linearity of the detector response was checked from this set of 6 working standards. Calibration graphs were prepared by plotting the peak area of biphenyl against the concentration.
3.2.1.3. Sample matrices

Refined glycerin, C₈₋₀ - C₁₀₋₀ methyl ester mixture, C₁₂₋₀ - C₁₄₋₀ fatty alcohol mixture, C₈₋₀ - C₁₀₋₀ fatty acid mixture, oleic acid, palm olein, corn oil and sunflower oil were used for spiking.

3.2.1.4. Recovery studies

5 replicate samples, each weighing 1 g, were introduced into five 10-mL volumetric flasks. Then 1, 0.5, 0.2 and 0.1 mL of the working standards (1 μg/mL) were added to the samples, and diluted to 10 mL with tetrahydrofuran to provide spiked solutions containing 1, 0.5, 0.2 and 0.1 μg/g of Dowtherm A. A minimum of three injections of 20 μL each were carried out for each sample, and the respective peak areas for biphenyl were obtained. Recoveries were calculated by interpolation from the calibration curve established earlier.

The LOD in the HPLC system was estimated from the representative blank samples. It was equated to the minimum concentration detectable when the signal-to-noise ratio (S/N) is at least 3:1. The limit of quantitation (LOQ) was equated to at least 10 times of the LOD¹¹⁹.

3.2.1.5. Collaborators

The laboratories that participated in the interlaboratory study were the Advanced Oleochemical Technology Center and the Product Development and
Quality Unit of the Malaysian Palm Oil Board (MPOB), and the Faculty of Food Technology of the University Putra Malaysia.

3.2.1.6. HPLC conditions

A reversed-phase column (250 mm x 4.6 mm i.d.) packed with 5 μm LiChrospher C<sub>18</sub> (GL Sciences Inc., Tokyo) was used in conjunction with a 50 mm x 4.6 mm i.d. guard column packed with same material. The system was run isocratically with a mobile phase of methanol and water (90:10, vol/vol). The flow rate was 1.0 mL/min, and the column was maintained at 35°C. The fluorescence detector was optimized at an excitation wavelength of 247 nm and emission wavelength of 310 nm.

3.2.1.7. GC-MS conditions

The column temperature was programmed from 150°C to 280°C at the rate of 5°C/min, and held at 280°C for 20 min. The injector and detector temperatures were maintained at 280°C.
3.2.2. Partially hydrogenated terphenyls

3.2.2.1. Materials

\( o-, m-, \) and \( p-\)Terphenyls were purchased from Fluka (Bellefonte, PA), and 1,4-dicyclohexylbenzene was purchased from Sigma (St. Louis, MO). Palm-based glycerin (99.5\%) and fatty acids (>99\%) such as \( \text{C}_{8:0}, \text{C}_{10:0}, \text{C}_{12:0}, \text{C}_{14:0}, \text{C}_{16:0}, \) and \( \text{C}_{18:0} \) were all provided by the oil refiners in the local oleochemical industry. Palm olein, sunflower oil, soybean oil and canola oil were also obtained from the local industry.

The thermal heating fluid (Thermolin 66\textsuperscript{TM}) was obtained from Solutia Inc. (St. Louis, MO).

3.2.2.2. Hydrogenation of terphenyls

The hydrogenation was conducted according to the procedure as reported by Scola \textit{et al.}\textsuperscript{120}. In each experiment, 50 g of terphenyl and 3.0 g powdered Harshaw 5256P nickel catalyst (Engelhard De Meern B.V., De Meern, The Netherlands) were introduced into the reaction chamber of a high-pressure reactor (Yamazaki Keiki, Tokyo). The reactor was pressurized with \( \text{H}_{2} \) to 20 kg/cm\(^3\) without stirring. Once the desired temperature (\textit{ca.} 200-250\textdegree C) was reached, stirring was started. The hydrogenation process was then carried out for 1-5 min at 50 kg/cm\(^3\), and the progress of the hydrogenation was monitored by following the pressure drop in the system. After the reaction mixture was allowed to cool to room temperature, a small
quantity of the product was dissolved in methanol and tetrahydrofuran (50/50, vol/vol). The solution thus obtained was filtered to remove the catalyst before analysis. The identity of the compounds was established by comparison with standards, and also with data reported by Scola et al.¹²⁰.

3.2.2.3. Analysis of glycerin and fatty acids

(a) Working solutions—Calibration solutions of Therminol 66™ (0.01 to 1.0 μg/mL) were prepared gravimetrically and dissolved in methanol and tetrahydrofuran (50:50, vol/vol), and 20 μL each was injected into the HPLC system in triplicate. A calibration graph was then obtained by plotting peak areas of 3- and 4-cyclohexylbiphenyl against the concentrations of Therminol 66™. (b) Recovery studies—A 1 g sample was accurately weighed into each of three 10-mL volumetric flasks. Then 1, 0.5 and 0.1 mL of the working solution (1 μg/mL) were added to the samples, and diluted to 10 mL with methanol and tetrahydrofuran (50:50, vol/vol), to provide spiked solutions containing 1, 0.5, and 0.1 μg/g Therminol 66™. A total of at least 3 injections of 20 μL each were carried out for each sample, and the respective peak areas of 3- and 4-cyclohexylbiphenyl were obtained. Recoveries were calculated by interpolation from the calibration graph established earlier. Sample blanks dissolved in methanol and tetrahydrofuran (50:50, vol/vol) were also analyzed as control.

The detection limits were examined by injecting standard solutions of progressively lower concentrations into the HPLC system until the signal with S/N ≥ 3 was obtained. The accuracy was determined as the difference between
calculated and actual concentrations of the spiked samples, and precision was assessed in terms of the coefficients of variation of the calculated concentrations in a replicate set\textsuperscript{121}.

3.2.2.4. Analysis of vegetable oils

(a) Working solutions—A 100-μg/mL stock solution was prepared by dissolving 10 mg Therminol 66\textsuperscript{TM} with ethanol in a 100-mL volumetric flask. The appropriate aliquots of the stock solution in ethanol were diluted to produce working standards with 10, 5, 1, 0.5, 0.1, and 0.01 μg/mL. Linearity of the detector was checked from this set of 6 working standards. Calibration graphs were prepared by plotting the peak areas of 3- and 4-cyclohexylbiphenyl against the concentrations of Therminol 66\textsuperscript{TM}. (b) Sample preparation—The blank sample (10% w/vol) was prepared by mixing 5 g of oil with 45 mL ethanol (95%) and 5 mL of aqueous KOH (50%, w/vol) in a 250-mL round-bottomed flask. The mixture was then saponified at about 80°C for 1 hr under reflux (11). A 20-μL of the cooled solution was analyzed directly.

To obtain the spiked oil sample with 1.0 μg/g Therminol 66\textsuperscript{TM} for recovery studies, 5 mL of the working solution (1 μg/mL) was added to a pre-weighed oil sample (5 g) in a 250-mL round-bottomed flask. The solution was further mixed with 40 mL ethanol and 5 mL aqueous KOH (50% w/vol). The spiked solutions containing 0.5 and 0.1 μg/g Therminol 66\textsuperscript{TM} were prepared in a similar manner except that 0.5 and 0.1 μg/mL of working solutions were used, respectively. The final concentration of oil mixture was 10% (w/vol), and 20 μL of each of these
solutions was analyzed by HPLC. Recoveries were calculated by interpolation from the calibration curve established earlier.

3.2.2.5. HPLC conditions

The system was run isocratically with a mobile phase of methanol and water (88:12, vol/vol). The flow rate was set at 1.0 mL/min, and a reversed-phase column (5 μm LiChrospher C_{18}, 250 x 4.6 mm i.d., GL Sciences Inc., Tokyo) was maintained at 40°C. The fluorescence detector was optimized at an excitation wavelength of 257 nm and emission wavelength of 320 nm.

3.2.2.6. GC-MS conditions

The column temperature was programmed from 150°C to 280°C at the rate of 5°C/min, and held at 280°C for 20 min. The injector and detector temperatures were maintained at 280°C.

3.2.3. Synthetic hydrocarbons

4.2.3.1. Materials

Palm-based glycerin (99.5%) and fatty acids (>99%) such as C_{12:0}, C_{14:0}, C_{16:0}, and C_{18:0}, palm olein, sunflower oil, soybean oil and canola oil were obtained locally. Alumina (Type WN-3: Neutral, activity Grade 1) for column
chromatography was purchased from Sigma (St. Louis, MO). The thermal heating fluid (Therminol 55\textsuperscript{TM}) was supplied by Solutia Inc. (St. Louis, MO).

3.2.3.2. Preparation of glassware

All washed glassware was rinsed with deionized distilled water and then dried in an oven at 220\textdegree C. After cooling, and just prior to use, the glassware was rinsed with acetonitrile followed by dichloromethane and hexane, the latter being allowed to evaporate with the aid of nitrogen stream before proceeding further.

3.2.3.3. Glass column chromatography

The preparative glass column (250 x 20 mm-i.d., with sintered glass disc and fitted with Teflon stopcock) was pre-filled with about 50 mL of hexane. By using a powder funnel, 50 g of dried alumina was introduced into the column. The column was then washed with additional 100 mL of hexane before the solvent level was allowed to fall to about 2-3 mm above the surface of the alumina.

3.2.3.4. HPLC conditions

The system was run isocratically with a mobile phase of ethanol and water (95:5, vol/vol). The flow rate was set at 0.8 mL/min, and a reversed-phase column (5 \textmu m LiChrospher C\textsubscript{18}, 250 x 4.6 mm i.d., GL Sciences Inc., Tokyo) was maintained at 40\textdegree C. The fluorescence detector was optimized at an excitation
wavelength of 270 nm and emission wavelength of 320 nm whereas the UV detector was at 262 nm.

3.2.3.5. GC-FID conditions

The oven temperatures were initially set at 70°C for first 5 min, then programmed to 280°C at the rate of 5°C/min, and finally held at 280°C for an additional 23 min. The injector temperature was set at 280°C while the detector was held at 290°C. The carrier gas was helium at the flow rate of 0.8 mL/min, and all analyses were performed using the splitless mode.

3.2.3.6. Quantitation

Calibration solutions of Therminol 55™ (10 to 1000 μg/mL) were prepared gravimetrically in hexane, and 100 μL each was injected into the HPLC system in triplicates. Quantitation of the fluid was based on the peak height counts at 10.9 min, and the baseline was accomplished by drawing a line parallel to the x-axis from 0 to 30 min.

The recovery study was carried out on spiked samples since contaminated samples were not available. A 10-g sample was accurately weighed into each of six 100-mL volumetric flasks, then 10 mL of each working solution (500, 100, 50, 30, 20 and 10 μg/mL) were added to the samples, and diluted to 100 mL with hexane for fatty acids and vegetable oils, whereas glycerin was dissolved in ethanol, to provide spiked solutions containing 500-10 μg/g Therminol 55™.
A total of 30 mL of the sample solution was pipetted into the alumina column which has been pre-conditioned. Once the sample solution has fallen to about 2-3 mm above the surface of the alumina, 200 mL of hexane was introduced gradually. The eluent was then collected and evaporated in a waterbath (60°C) to 1 mL under a gentle flow of nitrogen stream. 100 μL of the concentrate was then analyzed with HPLC while 1 μL was used for GC.

3.2.4. Diesel fuel

3.2.4.1. Reference chemicals

Tri-, di-, and mono-glycerides, palmitic acid, and α-tocopherol were purchased from Sigma Chemical Company (St. Louis, MO) and diesel samples were obtained from local petrol stations.

3.2.4.2. Standard solutions

A stock solution of diesel (1000 μg/mL) in heptane was prepared. Appropriate volumes of the stock solution were diluted in heptane to give working standards of 5, 10, 20, 50, 100, and 500 μg/mL. 1 g crude palm oil sample was accurately weighed into six 10-mL volumetric flasks, then 1.0 mL of each working standard was added to each of the 6 samples, and it was diluted to 10 mL with heptane. This provided spiked solutions containing 5 to 1000 μg diesel in 1.0 g edible oil. 20 μL of each solution was injected into the HPLC system.
3.2.4.3. Test samples

10 crude palm oil (CPO) samples from different local palm oil mills were used as blank references. Another 20 CPO samples from overseas, suspected to be contaminated by diesel, were used in the analysis. All samples were stored in a cold room until analysis. Prior to analysis, they were thoroughly mixed after warming.

3.2.4.4. Validation

Linearity of the method was demonstrated with different diesel oil standards at seven concentration levels over the range of 5-1000 μg/mL. Diesel concentrations were plotted against the peak height at 3.10 min and analyzed using simple linear regression. 1 g of each edible oil sample was weighed into a 10-mL volumetric flask and diluted with heptane to the mark. Triplicate injections of 20 μL each were performed, and the peak heights at 3.10 min were obtained. The recoveries were calculated by interpolation from the calibration curve established earlier.

3.2.4.5. Glass column chromatography

The column (800 mm x 40 mm i.d.) was packed with about one-third of premixed silica gel slurry in hexane. Approximately 30 g CPO, both blank and contaminated samples, was loaded onto the adsorbent and eluted with an additional 500 mL of hexane. The eluate was collected in a 500-mL flask and evaporated in a
gentle stream of nitrogen with the flask at 60°C (water bath). The residue was redissolved with 1 mL hexane, and 1 μL was injected into the GC-mass spectrometer.

3.2.4.6. HPLC conditions

Separation was performed at 35°C on an Apex Diol II (250 mm x 4.6 mm i.d., 5 μm particle size; Jones Chromatography Ltd., Mid Glamorgan, England). The isocratic mobile phase consisted of heptane and isopropanol (94:6, vol/vol) with a flow rate of 1.0 mL/min. The fluorescence detector set at 286 nm (excitation) and 321 nm (emission) was connected in series with an ELSD (nebulizer tube was set at 60°C and 2.3 bar air pressure).

3.2.4.7. GC-MS conditions

Temperature programming for the column was employed using the following parameters:- at 70°C for first 5 min, then heated at 15°C/min from 70 to 280°C and held for 20 min at the final temperature. The injector and detector temperatures were set at 250°C.