Chapter 2

Experimental

2.1 Chemicals and materials

2.1.1 Standard chemicals

- Di (2-ethylhexyl) phthalate (DEHP) (purity 97%, GC Grade, Fluka, Switzerland)
- Di (2-ethylhexyl) adipate (DEHA) (purity 97%, GC Grade, Fluka, Switzerland)

2.1.2 General chemicals and solvents

- Acetone (CH_3COOCH_3, AR grade: Merck, Germany)
- Cyclohexane ($CH_2(CH_2)_4CH_2$, AR grade: Carlo Erba, USA)
- Dichloromethane (CH_2Cl_2, AR grade: Merck, USA)
- Ethyl acetate ($CH_3COOC_2H_5$, AR grade: LAB-SCAN, Thailand)
- Florisil® 60-100 mesh ($Mg_3Si_4O_{10}$: Merck, Germany)
- Glass wool
- n-Hexane (C_6H_{14}, AR grade: Merck, Germany)
- Methanol (CH_3OH, AR Grade: LAB-SCAN, Thailand)
- Sodium sulphate anhydrous (Na_2SO_4, AR grade: Merck, Germany)
- Ultra pure water (H_2O, Synthesis in laboratory by Maxima, ELGA, England)

2.1.2 Samples

Packaged food samples were purchased from Department Stores in Songkhla province.

2.2 Instruments and apparatus

2.2.1 Gas chromatography- Flame Ionization Detector (GC- FID)

- Gas chromatograph model GC 8960 series equipped with flame ionization detector (Agilent, USA.)
- Capillary Column: 30 m × 0.25 mm I.D., 0.25 μm film thickness of 5% phenyl 95% dimethylpolysiloxane, HP-5MS (Agilent, USA)
- Computer system model VL Vectra (Hewlette Packard, USA)
- Chemstation software (Agilent, USA)
- High purity helium carrier gas (purity 99.995% (TIG, Thailand)
- Oxidant gas, Air zero grade (purity 99.995%: TIG, Thailand)
- High purity nitrogen gas (purity 99.995%: TIG, Thailand)
- High purity hydrogen gas (purity 99.995%: TIG, Thailand)
- Flow meter (Agilent, USA)
- Solid Phase Extraction (SPE)
- Florisil®cartridges (Florisil® 1 g in 2 mL glass syringes)
- SPE vacuum manifold (Supelco, USA.)
- Vacuum pump (Gast manufacturing, Inc., USA)

2.2.2 Apparatus

- Analytical balance (Sartorius, USA)
- Amber vial 2 mL with septum and silver aluminum cap (Agilent Technologies, USA)
- Buchner funnel (7.0 cm i.d. × 10.1 cm high)
- 11-mm crimper and 11-mm decrimper (Agilent Technologies, USA)
- Dessicator
- Evaporating rotator (BUCHI, Germany)
- Filter paper-No.42 (7 cm i.d., Whatman, Maidstone, England)
- Glass microfibre filter paper GF/F 70 mm (Whatman, Maidstone, England)
- General glasswares such as volumetric flask 5, 10, 50 mL; round bottom 50 mL, test tubes, beakers
- Glass syringe (0.95 cm i.d. × 6.8 cm high)
- Microliter pipette: 10 μL, 20 μL, 200 μL, 1000 μL (Eppendorf, Germany)
- Measurement pipette 1, 2, 5, 10 mL (Pyrex, USA)
- Oven (Fisher Scientific, UK)
- Refrigerator (Hitachi, Japan)
- Syringe Filter, Filter device PVDF filter media with polypropylene housing, 13 mm. i.d., 0.2 μm pore size (Chrom Tech, USA)
- Syringe for sample filter, Micro-Mate® interchangeable (Popper & Son, Inc, ITALY)
- Syringe, 10 μL (Agilent Technologies, USA)
- Syringe cleaner (Hamilton, USA)
- Ultrasonic bath (Automatic science, Thailand)
- Vibrator (Dremel Engraver, USA)

- Vortex (Genei Scientific Industries Inc., USA)

2.3 Analysis system

In this work di (2-ethylhexyl) phthalate (DEHP) and di (2-ethylhexyl) adipate (DEHA) in packaged food were analyzed by gas chromatographic technique with flame ionization detector (GC-FID). An overview of the analysis system is shown in Figure 4. It consists of two parts: sample preparation and analysis. The sample preparation part consists of an ultrasonic bath, an evaporating rotator, an SPE vacuum manifold and a vacuum pump. These were used for ultrasonic extraction and solid phase extraction (SPE) using Florisil® cartridges. A Hewlett Packard gas chromatograph (GC, Agilient 6890 series) coupled with flame ionization detector was used for the analysis. Analytes were separated on a HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness).

To obtain the highest efficiency, parameters affecting the performance of the GC-FID and sample preparation were optimized (see 2.5 and 2.8). The methods for analysis of phthalate and adipate esters were validated (2.11). Finally, real samples were collected, extracted and analyzed by GC-FID at optimum conditions.

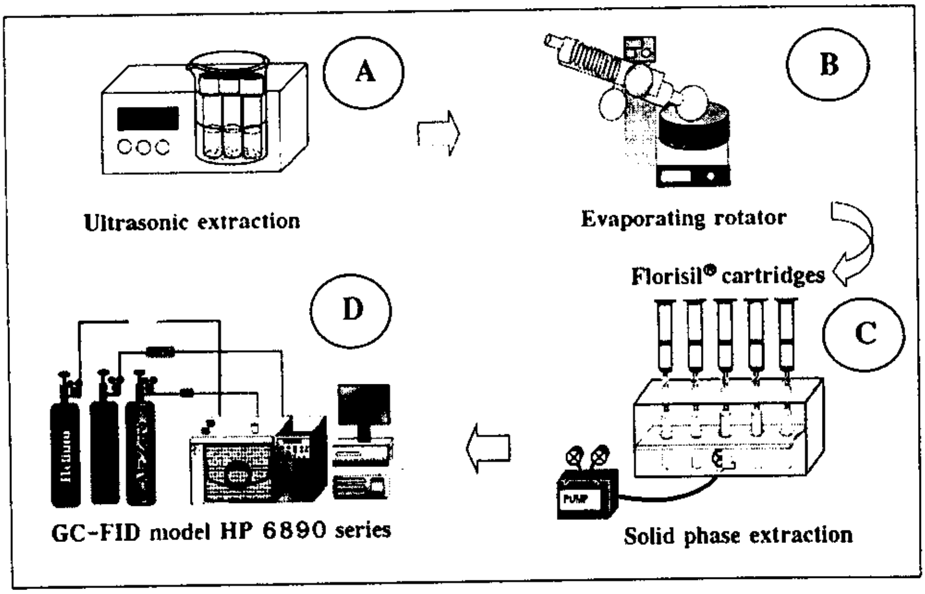

Figure 4 The analysis system for DEHA and DEHP analysis
A, B and C represent a sample preparation part
D represent an analysis part

2.4 Standard solution

In the analysis of phthalate ester high blank signals are often encountered due to the presence of phthalate in many laboratory products, including chemicals and glasswares. To avoid phthalate contamination, all glasswares were washed with detergent, rinsed with distilled water, hexane, and acetone, then dried at 140 °C for at least 4 hours (Shen, 2005).

2.4.1 DEHP-DEHA standard stock solution

Standard stock solution of DEHP-DEHA, 1000 µg mL^{-1} each, were prepared by pipetting 26.2 µL of DEHP and 27.9 µL of DEHA into a 25 mL volumetric flask and adjust the volume with ethyl acetate. Intermediate solution was prepared by diluting the standard stock solution to the concentration of 100 µg mL^{-1} in ethyl acetate. All standard solutions were transferred into glass bottles with polytetrafluoroethylene (PTFE)-lined screw caps, wrapped with aluminum foil to protect from light and stored at 4 °C. The bottles were brought to room temperature prior to use. Stock standard solutions were checked periodically by gas chromatograph for signs of degradation or evaporation, especially just prior to preparation of calibration standards (EPA method 8061A, 1996).

2.4.2 DEHP-DEHA working standard solution

DEHP-DEHA working standard solutions used in the experiments were prepared as mixture of DEHP and DEHA by diluting stock standard solution with ethyl acetate to obtain the solution with a concentration of 10 $\mu g\ mL^{-1}$. The solution was used to test the optimum conditions.

2.5 Optimization of GC-FID conditions

The investigated conditions of the GC-FID system for analysis of phthalate and adipate esters were carrier gas flow rate, column temperature programming, injector temperature, detector temperature, fuel gas (H_2) flow rate, oxidant gas (Air) flow rate. The optimum conditions were obtained by considering the best resolution, highest response and less analysis time. In these studies, 1 μL of DEHP-DEHA working standard solution, 10 $\mu g\ mL^{-1}$, was injected into the gas chromatograph for analysis. Optimizations were carried out by varying one parameter at a time while the others were kept constant. Five replications were performed for each tested value. The starting operating conditions of GC-FID system were as shown in Table 4. When an optimize value of one parameter was obtained it was used in the optimization of the next parameter following the sequence in this section.

Table 4 GC-FID starting operating conditions (Agilent, 1995; Simonean and Hannaert, 1999)

Column: Capillary column	HP-5MS, 30 m × 0.25 mm I.D., 0.25 μm film thickness of 5% diphenyl 95% dimethylpolysiloxane
Flow rate: He, carrier gas	1.0 mL min^{-1}
H_2, fuel gas	30 mL min^{-1}
N_2, make up gas	30 mL min^{-1}
Air, oxidant gas	300 mL min^{-1}
Column temperature program:	
Initial temperature	100 °C
Initial hold time	0 minute
Ramp rate	20 °C min^{-1}
Final temperature	300 °C
Final hold time	4 minutes
Injector temperature	265 °C
Detector temperature	300 °C

2.5.1 Carrier gas (He) flow rate

To optimize the carrier gas flow rate 1 μL of 10 μg mL^{-1} DEHP-DEHA standard solution was injected to the GC-FID system. Nitrogen, hydrogen and air were used as make up, fuel and oxidant gases and were initially maintained at flow rates of 30, 30 and 300 mL min^{-1}, respectively. These values were suggested in the Operating Manual of GC-FID model HP 6890 series. Optimum carrier gas flow rate was investigated by varying the flow rate at 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8 mL min^{-1}. The retention time and peak area from chromatograms of DEHP and DEHA were used to calculate the plate number (*N*) and high equivalent to a theoretical plate (HETP). From the van Deemter plot, the optimum flow rate was obtained from the lowest HETP.

2.5.2 Column temperature programming

Column temperature programming consists of the following steps: initial temperature, hold time of the initial temperature, ramp rate, final temperature and hold time of the final temperature. The optimum column temperature program was obtained by considering the best resolution, highest response and less analysis time. To optimize the column temperature programming parameters 1 μL of 10 μg mL^{-1} DEHP-DEHA standard working solution was injected into the GC-FID system by varying one parameter at a time as follows.

Step I - Initial temperature

The optimum initial temperature was investigated by varying the temperature at 90, 100, 110, 120, 130, 140, 150 and 160 °C. The ramp rate was 20 °C min^{-1} and the final temperature was 300 °C.

Step II - Hold time of the initial temperature

The hold time of initial temperature was varied at 0, 1, 2, and 3 minutes. Other parameters were set as, injector temperature 265 °C, detector temperature 300 °C and the column temperature programming was 110 with ramp rate at 20 °C min^{-1} to final temperature at 300°C and hold for 4 minutes.

Step III - Ramp rate of temperature

The ramp rate was varied at 10, 15, 20, 25 and 30 °C min^{-1}.

Step IV - Final temperature

Optimum final temperature was investigated by varying the temperature at 260, 270, 280, 290 and 300 °C.

2.5.3 Injector temperature

The optimization of injector temperature was investigated by varying the temperature at 235, 245, 255, 265, 275, 285 and 295 °C. The temperature that gave the highest response was selected.

2.5.4 Detector temperature

The optimization of detector temperature was investigated by varying the temperature at 260, 270, 280, 290 and 300 °C. Responses obtained from the different temperatures were compared. The temperature that provided the highest response was the optimum detector temperature.

2.5.5 Fuel (H_2) flow rate

The flow rate of air (oxidant) was set at 300 mL min^{-1}, the recommended flow rate in the Operating Manual of GC-FID model HP 6890 series. Hydrogen was used as the fuel gas and its flow rate was investigated at 20, 30, 40 and 50 mL min^{-1}. The peak areas obtained from all flow rates were compared and the optimum oxidant flow rate was determined from the highest response.

2.5.6 Oxidant (air) flow rate

Optimum oxidant gas flow rate was investigated at 100, 200, 300 and 400 mL min^{-1}. The peak areas obtained from all flow rates were compared and the optimum oxidant flow rate was determined from the highest response.

2.6 Limit of detection (LOD)

Standard mixture of DEHP and DEHA at a concentration of 10 μg mL^{-1} was diluted with ethyl acetate to obtain the concentration in the range of 12 ng mL^{-1} to 0.5 μg mL^{-1}. A 1 μL aliquot was injected into the GC-FID system at the optimum conditions (2.5.1-2.5.6). The signal, peak area, was used to calculate the signal to noise ratio (S/N). The lowest concentration or amount of analytes that the flame ionization detector could detected and has a signal to noise ratio greater than 3 was taken as the detection limit according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (ICH Q2B guidance "Validation of Analytical Procedures", 1996).

2.7 Linear dynamic range (Linearity)

Standard mixture solutions of DEHP and DEHA were prepared at concentrations between 25 ng mL^{-1} to 60 μg mL^{-1}. A 1 μL aliquot of each concentration was injected into the GC-FID system that was set at the optimum conditions from 2.5.1-2.5.6. Linear dynamic range of the response was determined by plotting the calibration curve. The linearity of the response was determined by considering the linear regression coefficient.

2.8 Sample preparation

2.8.1 Adsorbent conditioning

The adsorbent used in this study was Florisil®, 60-100 mesh (Figure 5). Florisil®, a registered trade name of U.S. Silica Co., is an activated magnesium silicate with basic properties. It is used to separate analytes from interfering compounds prior to sample analysis by a chromatographic method (EPA method 3620B, 1996). The physical properties of Florisil are shown in Table 5.

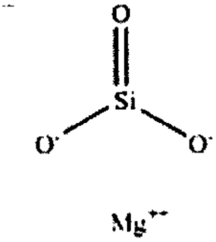

Figure 5 The chemical structure of Florisil

Table 5 Physical properties of Florisil® 60-100 mesh

Formula structure	MgO_3Si
Molecular weight	100.39 g moL^{-1}
Specific Gravity	2.51 g mL^{-1}
Mesh size	60/100 mesh
Surface area	289 m^2 g^{-1}
Odor	Odorless

Before use, it was recommended that Florisil® must be deactivated with water to clean up the phthalate esters that might come with it. These was done by placing 100 ± 10 g of Florisil® into a 500 mL beaker and heated to 140 °C for approximately 16 hours. After heating, it was transfered to a 500 mL reagent bottle, tightly sealed and cooled to room temperature. When cool, 3.0 ± 0.1 mL of ultra pure water was added and it was mixed thoroughly by shaking or rolling with glass stirring rod for 10 minutes and was left to stand for at least 2 hours. It was kept in a glass bottle, wrapped with aluminum foil (EPA method 3620B, 1996) and stored in a cool, dry place, to protect against physical damage (Material Safety Data Sheet, 2004)

2.8.2 Preparation of Florisil® cartridges

A Florisil® cartridge was prepared by first plugged a 0.9 cm inner diameter of GF/F glass microfibre filter into the bottom of a 2 mL glass syringe (0.9 cm i.d. × 6.8 cm high) using a syringe plunger followed by 1.00 g of Florisil® (60-100 mesh). The cartridge was moderately vibrated to settle the adsorbent then a GF/F glass microfibre filter was plugged on top as frit to retain the adsorbent. The packed Florisil® cartridge is shown in Figure 6.

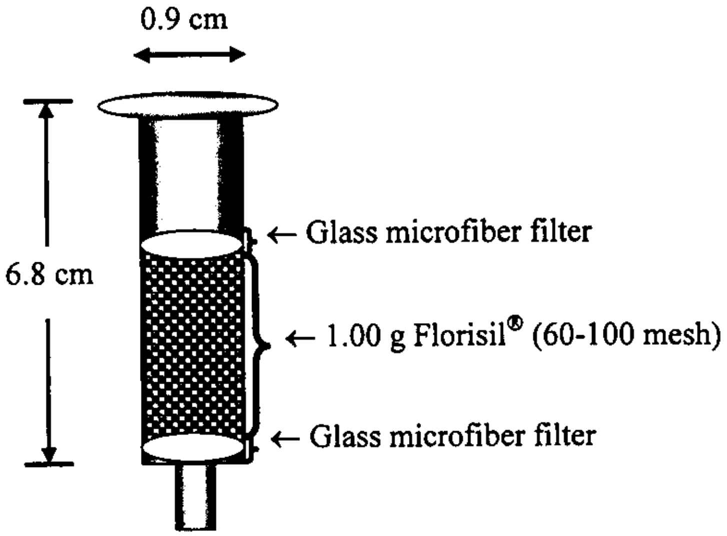

Figure 6 Packed Florisil® cartridge

2.8.3 Optimization of sample preparation procedure

This work proposed the combine use of ultrasonic extraction and solid phase extraction (SPE) for sample preparation. Sour yellow curry paste was used as a representative in the optimization of extraction conditions. For ultrasonic extraction, 3.00 g of curry paste sample was placed into a test tube and spiked with 30 μL of 1000 μg mL^{-1} standard (DEHP and DEHA) mixture solution. The solvent was llowed to evaporate for 15 minutes before proceeded to the sample extraction step.

The extraction solvent was added to the sample before closing the top of the test tube with a lid. Then placed the test tube in a 1000 mL beaker that contained some water and placed in an ultrasonic bath at room temperature. The combined solvent extract was filtered through a No. 42 filter paper in a filter funnel using gentle suction and dried with small quantity of anhydrous sodium sulphate. This was transferred to a 50 mL round bottom flask and evaporated just to dryness by a rotary evaporator at 55 °C and re-dissolved with 5 mL ethyl acetate. The ethyl acetate mixture was passed through a 0.2 μm polyvinyllidene fluoride filter (PVDF) in a 25 mL vial to remove any remaining curry paste residue. The ethyl acetate mixture was then used in the SPE method. The optimization of ultrasonic extraction *i.e.*, extraction time, extraction solvent and volume of extraction solvent were as described in 2.8.3.1-2.8.3.3.

For the SPE method, Florisil® cartridges were arranged on the manifold in a closed-valve position (Figure 7). The cartridges were conditioned by adding 12 mL of methanol followed by 12 mL of dichloromethane/hexane 4:1 (v/v). This was to eliminate all contaminations and to ensure that the sample solution properly wet the surface of the sorbent particles (Fifield and Kealey, 2000). After the removal of the solvents, each cartridge was then equilibrated with 1 mL of ethyl acetate. A few drops per cartridge was allowed to pass through the manifold to remove air bubbles. The eluent from the condition and equilibration step was then discarded. A Supelco Visiprep SPE vacuum manifold, with a vacuum pump set at 10 in Hg, was used to manipulate eluent flow rate. The cartridge valve was first opened to allow the ethyl acetate mixture (containing DEHP and DEHA) to pass through the sorbent bed into collection test tubes. The valves were closed to remove the collection test tubes (containing DEHP and DEHA) and opened again to dry Florisil® cartridges under vacuum (10 in. of Hg). The remaining DEHP and DEHA that were adsorbed by the Florisil® were eluted by the eluting solvent. An aliquot of the sample extractant (from loading and eluting steps) was then transferred into a round bottom flask and was evaporated just to dryness by a rotary evaporator at 55 °C and reconstituted with ethyl acetate to a final volume of 2 mL. Finally, 1 μL was injected into the GC-FID system at optimum conditions. The optimization of solid phase extraction for analysis of phthalate and adipate esters *i.e.*, sample flow rate, type of eluent solvent, volume of eluting solvent, drying time, flow rate of eluting solvent were as described in 2.8.3.4-2.8.3.8. The summary of the sample preparation procedure is shown in Figure 8.

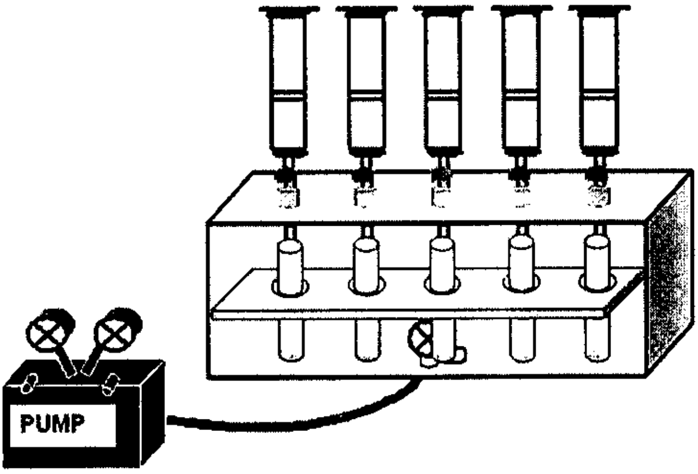

Figure 7 Solid phase extraction of Florisil® cartridges

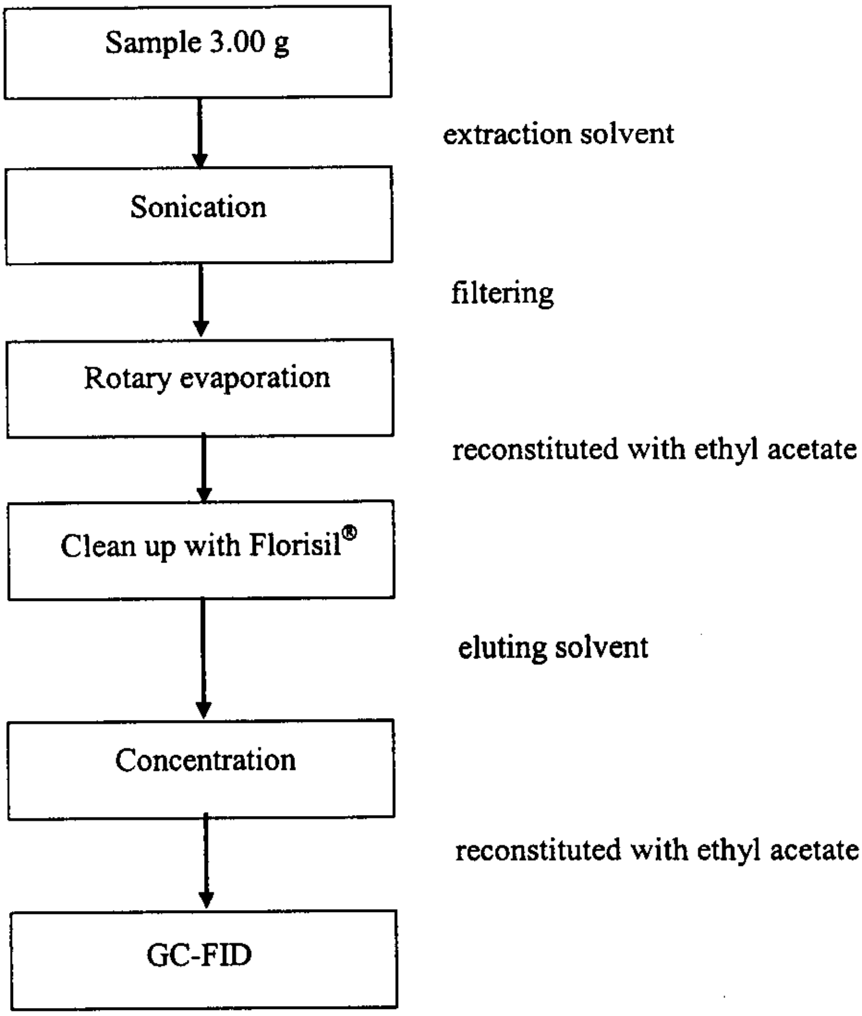

Figure 8 Analytical procedure of phthalate and adipate esters for curry paste samples

The extraction parameters of curry paste samples were optimized and were repeated five times with each parameter. Five replications of each extractant were analyzed with GC-FID at optimum conditions. The starting operating conditions for the optimization of sample preparation are shown in Table 6. When an optimized value of one parameter was obtained it was used in the optimization of the next parameter.

Table 6 The starting operating conditions for the optimization of sample preparation

Steps	Parameters	Starting operating values
I	Extraction time	60 minutes
II	Extraction solvent	dichloromethane-cyclohexane 1:1 (v/v)
III	Volume of extraction solvent	20 mL
IV	Sample flow rate	1 mL min^{-1}
V	Drying time	6 minutes
VI	Type of eluting solvent	dichloromethane-hexane (4:1) (v/v)
VII	Flow rate of eluting solvent	1 mL min^{-1}
VIII	Volume of eluting solvent	12 mL

2.8.3.1 Extraction time

Extraction time of the ultrasonic step is one parameter influencing the extraction of analytes. To ensure the accuracy of the results it was necessary to completely release DEHP and DEHA from the sample matrix during the ultrasonic extraction. Good extraction method should provide high extraction efficiency in a short period of extraction time. To check the influence of the extraction time, the sample was extracted in 20 mL of cyclohexane-dichloromethane 1:1 (v/v) and the time was varied at 30, 60, 90, 120 and 150 minutes. The extraction time that provided the highest response was selected.

2.8.3.2 Extraction solvent

The extraction solvent should be highly selectivity to analytes. Five extraction solvents for DEHP and DEHA extraction were investigated. The polarity of the solvent must be considered for the highest extraction efficiency.

These extraction solvents were selected from literatures such as hexane-acetone 1:1 (v/v) (EPA method 8061A, 1996), hexane-dichloromethane, 1:1 (v/v) (Mackintosh *et al.*, 2004), dichloromethane-cyclohexane 1:1 (v/v) (Lau and Wong, 1996), acetonitrile (Tsumura *et al.*, 2002) and ethyl acetate (Niino *et al.*, 2002). The solvent that provided the best and highest response extraction efficiency was selected.

2.8.3.3 Volume of extraction solvent

From the result in experiment 2.8.3.2 dichloromethane-cyclohexane 1:1 (v/v) was selected as extraction solvent to extract analytes from sample matrix. For a good extraction the volume of the extraction solvent should be enough to extract the analytes in order to obtain the highest yield. The volume of extraction solvent was varied at 10, 20, 30, and 40 mL and the one that provided the best response was selected.

2.8.3.4 Sample flow rate

The flow rate of sample through the SPE cartridge was optimized for the maximum efficiency. The flow rate was varied at 1, 3, 5 and 7 mL min^{-1}. The flow rate that gave the highest response was then selected.

2.8.3.5 Drying time

Since the elution solvent is water-immisible it was important to remove the residual water from the SPE cartridge that should be dried prior to the elution of DEHP and DEHA. The drying time was varied at 0, 3, 6 and 9 minutes and the drying time that gave the highest response was then selected.

2.8.3.6 Type of eluting solvent

The elution solvent should be strong with respected to the analytes for high selectivity. Five elution solvents for extraction of DEHP and DEHA from Florisil® were studied, These elution solvents were selected from literatures *i.e.*, ethyl acetate (Niino *et al.*, 2003), 50/50 (v/v) hexane-diethylether (Ma *et al.*, 2003), 10/90 (v/v) acetone-hexane (Sablayrolles *et al.*, 2005), dichloromethane-cyclohexane 1:1 (v/v) (Mackintosh *et al.*, 2004) and dichloromethane-hexane 4:1 (v/v) (Casajuana and Lacorte, 2004). The eluting solvent that provided the best and highest response extraction efficiency was selected.

2.8.3.7 Volume of eluting solvent

From the result in experiment 2.8.3.5, 10/90 (v/v) acetone-hexane was selected as eluent to elute analytes from Florisil® cartridge. To minimize solvent used and at the same time maintain the high response, the volume of 10/90 (v/v) acetone-hexane was studied by varying at 1, 3, 5, 7, 9 and 12 mL. The least solvent volume that provided the highest was then selected.

2.8.3.8 Flow rate of eluting solvent

The analytes were eluted from Florisil®, using the mixture of acetone-hexane (10/90, v/v). In order to minimize solvent used and at the same time maintained the high response, the flow rate of eluting solvent was varied at 1, 3, 5 and 7 mL min^{-1}. The flow rate that gave the highest response was then selected. The summary of the optimization for sample preparation is shown in Table 7.

Table 7 Optimization of sample preparation

Steps	**Parameters**	**Optimization values**
I	Extraction time	30, 60, 90, 120 and 150 minutes
II	Extraction solvent	hexane-acetone 1:1 (v/v), hexane-dichloromethane 1:1 (v/v), dichloromethane-cyclohexane 1:1 (v/v), acetonitrile and ethyl acetate
III	Volume of extraction solvent	10, 20, 30 and 40 mL
IV	Sample flow rate	1, 3, 5 and 7 mL min^{-1}
V	Drying time	0, 3, 6 and 9 minutes
VI	Type of eluting solvent	ethyl acetate, 50/50 (v/v) hexane-diethylether, 10/90 (v/v) acetone-hexane, dichloromethane-cyclohexane 1:1 (v/v) dichloromethane-hexane 4:1 (v/v)
VII	Flow rate of eluting solvent	1, 3, 5, 7 mL min^{-1}
VIII	Volume of eluting solvent	1, 3, 5, 7, 9 and 12 mL

2.9 Sampling

The samples were different types of curry paste packed in plastic packaging. Five common and popular types of curry paste were investigated, *i.e.*, Sour yellow curry paste, Red curry paste, Green curry paste, Masman curry paste and Panang curry paste (Table 8). All samples were sampling from supermarkets in Hat Yai district and stored at 4 °C until being analyzed.

Table 8 Curry paste samples for phthalate and adipate esters determination

Type of samples	Number of Sample	Samples	Date of expire
Sour yellow curry paste	2	1	22-12-06
		2	29-08-05
Red curry paste	2	1	16-02-07
		2	23-07-06
Green curry paste	2	1	19-05-07
		2	10-03-07
Masman curry paste	2	1	07-09-06
		2	06-01-07
Panang curry paste	2	1	17-02-07
		2	10-03-07
Total samples	10		

2.10 Matrix Interference

Matrix interference or matrix effect plays an important role in the accuracy and precision of a measurement. The sample matrix may lead to either a suppression or enhancement of the sample signal compared to the calibrant signal for the same analyte quantity (Roper *et al.*, 2001). Sample preparation steps are often sensitive to the matrix. Matrix spikes are used to determine their effect on sample preparation and analysis. Matrix spiking is done by adding a known quantity of a component that is similar to the analyte but not present in the sample originally. The sample is then analyzed for the presence of spiked material to evaluate the matrix effects (Mitra, 2003). In this work, the effect of matrix was studied by spiking known amount of DEHP-DEHA standard solution in the concentration range of 0.50 - 10 μg mL^{-1} in 3.00 g of curry paste samples before the extraction steps as describe in 2.8.3.

A sample blank was analyzed with each set of curry paste samples. A 1 μL aliquot of each extractant was injected into GC-FID system at optimum conditions. Each experiment was done in five replicates. The responses, peak areas, were plotted against the known concentrations. The slope of the standard and the spiked sample were compared for matrix interference and confirmed/evaluated by using the statistic test.

2.11 Method validation

In order to create confidence in the analysis procedures and complement enforcement requirements, methods for phthalate and adipate esters analysis need to be validated. The objective of the method validation is to demonstrate that the various steps in the analytical procedure, and may be valid for a restricted matrix, produce acceptably accurate and precise results for a given analyte. Five types of curry paste samples were used in the studies validating the method.

2.11.1 Recovery

The recovery of the method was performed by spiking curry paste samples with DEHP-DEHA standard solutions, 0.5 and 5.0 μg mL^{-1}. The spiked samples were left to stand for 15 minutes before extracted by applying the method described in 2.8.3. A blank curry paste was also extracted. A 1 μL aliquot of each extractant was injected into GC-FID system at optimum conditions. Five replicates were done in each concentration. The results from the extracted curry paste samples were compared with DEHP-DEHA standard solution prepared at the same concentrations. Percentage recovery (%R) is calculated as follow:

$$\%R = [(CF-CU)/CA] \times 100$$

Where CF is the concentration of analyte measured in the fortified sample or spiked sample.

CU is the concentration of analyte measured in the unfortified sample or blank.

CA is the concentration of analyte added in the sample.

(Eurachem, 1998).

2.11.2 Method detection limit (MDL)

The method detection limit (MDL) can be defined as the smallest amout of an analyte that can be reliably detected or differentiated from the background for a particular matrix (Corley, 2002). In this work, the method detection limit was investigated by spiking DEHP-DEHA standard solution into five types of curry paste samples to make final concentrations in the range 27 to 250 μg mL^{-1}. They were then extracted by the sample preparation procedure described in 2.8.3. A 1 μL aliquot of each extractant was injected into GC-FID system at optimum conditions. Five replicates were done for each concentration. A sample blank was analyzed with each set of curry paste samples to measure background contamination and the average blank was substracted from the analytes peak areas in spiked samples. A signal to noise ratio (S/N) more than 3 calculated from the chromatogram was considered acceptable for estimating the method detection limit according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (ICH-Q2B guidance "Validation of Analytical Procedures", 1996).

2.11.3 Limit of quantitation (LOQ)

Limit of quantitation of the method can be defined as the smallest amout of an analyte that can be reliably quantified with a certain degree of reliability within a particular matrix. It can be applied to analytical procedures that exhibit baseline noise. Determination of the signal to noise ratio was performed by comparing measured signals from samples with known low concentrations of analytes with those of blank samples and by establishing the minimum concentration at which the analytes can be reliably quantified. A signal to noise ratio (S/N) of 10 was considered acceptable for estimating the limit of quantitation (ICH Q2B, 1996).

2.11.4 Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation for a statically significant number of samples (Swartz *et al.*, 1997). To assess the precision of the method for the determination of DEHP and DEHA. Five types of curry paste samples were evaluated at two spiking levels of 0.5 and 5.0 μg mL^{-1} in 3.00 g of curry paste samples followed by sample preparation procedure described in 2.8.3. A sample blank was also analyzed in parallel. A 1 μL aliquot of each extractant was injected into GC-FID system at optimum conditions. Five replicates were performed at each concentration. The relative standard deviation (RSD) were then calculated for each type of samples by the following equations (Miller and Miller, 2000).

$$\%RSD = \frac{s}{\bar{x}} \times 100$$

$$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

Where s is the standard deviation

n is the total number of measurements

$\bar{x}$ is the mean of n measurements

2.12 Qualitative and quantitative analysis of phthalate and adipate esters in packaged food

2.12.1 Qualitative analysis

Qualitative analysis was carried out by comparing the retention time (t_R) of the chromatogram of DEHP-DEHA standard to unknown samples. The retention time is the time elapsed from injection of the sample component to the recording of the peak maximum (Grob, 2004).

2.12.2 Quantitative Analysis

Quantification of phthalate and adipate esters in packaged food was based on the response of chromatographic peak that was proportional to the amount of analyte. Two analytical techniques, matrix match calibration curve and the standard addition method were implemented in this work.

2.12.2.1 Matrix match calibration curve

Matrix match calibration curve was carried out by spiking all the analyzed compounds into 3.00 g of curry paste samples to the final concentration at 0.05, 1.0 , 5.0 and 10.0 μg mL^{-1}. The spiking was done before the sample preparation step. Then the spiked and unspiked samples were analysed chromatographically under the optimum conditions. The experiments were done in five replicates. The matrix match calibration curves were obtained by plotting the peak area, after subtracting the concentration of the analytes in the unspiked sample, versus concentration of DEHP and DEHA. The response from the chromatogram per unit of concentration of DEHP and DEHA in the unknown samples is then calculated mathematically from the matrix match calibration curve.

2.12.2.2 Standard addition method

In the case where concentration of analytes in the real samples was lower than method detection limit (MDL), the technique of standard addition was used. For standard addition method, the matrix is constant for all samples (Harris, 1948). A practical approach to standard addition is to devide the sample into several equal portions, then add increasing levels of standard. This method requires a linear response of the analyte . In this work, the curry paste samples were extracted following the sample preparation procedure described in 2.8.3. A constant volume (V_{unk}) 200 μL, of the unknown sample solutions (extractants) were added to each of the four 2 mL vials. Then 50 μL of DEHP-DEHA standard solution (V_{std}) at various concentrations were added to the sample solutions to make four different concentration levels at 30, 60, 90 and 120 ng mL^{-1}. The solutions were thoroughly mixed using a vortex. The resulting solution was injected to the GC-FID at optimum conditions. The experiments were done in five replicates. After measuring the response for a series of standard additions, the results were plotted between peak area and concentration of phthalate and adipate esters. The original concentration is then determined by extrapolation to the x-axis (Grob, 2004) as shown in Figure 9.

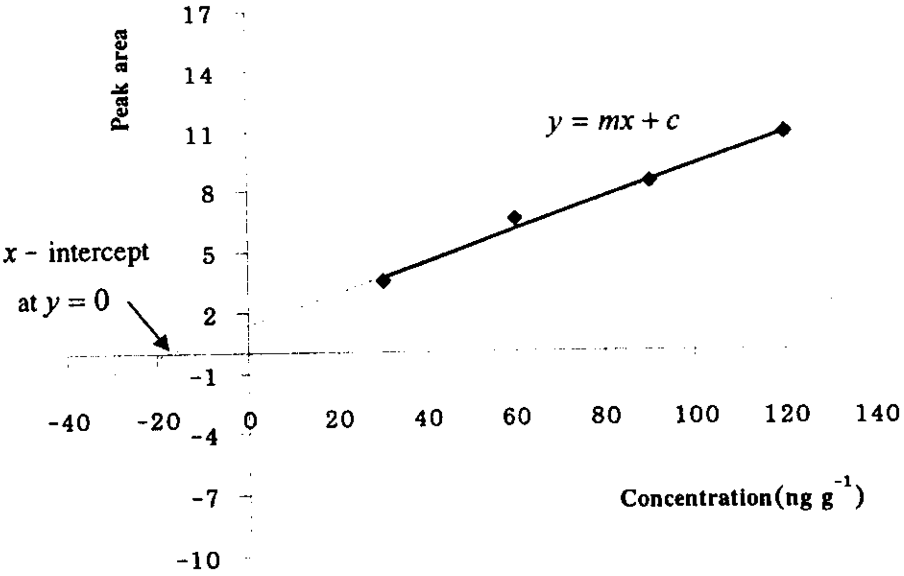

Figure 9 Standard addition method

2.13 Quality assurance and quality control

In the laboratory, at any stage of sample preparation and analysis, contamination can occur. Therefore, it must be demonstrate that during the sample preparation procedure it is essentially free of trace phthalate and adipate esters residue. This was assessed by preparing reagent blanks and method blank.

Reagent blanks are reagents used during the analytical process (including solvents used for extraction or dissolution). These are analysed in isolation in order to see whether they contribute to the measurent signal. The measurent signal arising from the analyte can then be corrected accordingly (Eurachem, 1998). Reagent blanks were prepared and analyzed for phthalate and adipate esters prior to the sample preparation steps.

Method blank is performed by carried through all the steps of sample preparation and analysis as if were an actual sample. It is used to evaluate the laboratory contamination for the targeted analytes and corrected systematic errors due to impurities that could arise from the reagents, the glasswares, or the laboratory environment (Harvey, 2000).