Extraction of Organochlorine Pesticides from Honey using Dispersive Liquid-liquid Microextraction Technique and Determined by Gas Chromatography-electron Capture Detector

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Abstract

An effective sample pretreatment method namely dispersive liquid-liquid microextraction (DLLME) has been utilized for matrix removal from honey samples. The DLLME procedure in this study was carried out using toluene as an extraction solvent and acetonitrile as a dispersive solvent mixed in a centrifuge tube. Additionally, the analytical method of two types of organochlorine pesticides including lindane and dieldrin using gas chromatography equipped with electron capture detector (GC-ECD) has been validated for an effective quantification of these pesticides in honey samples. The parameters of validation such as linearity, precision, limit of detection, limit of quantification and recovery were evaluated. The experimental results presenting the linearity for lindane and dieldrin data (R^2 values ≥ 0.995) were in the range of 0.05-1000 ppb and 1-3000 ppb, respectively. The limit of detection (LOD) values of lindane and dieldrin were 0.18 and 0.27 ppb and the limit of quantification (LOQ) values were 0.60 and 0.87 ppb, respectively. The repeatability values expressed in terms of relative standard deviation (%RSD) ranged from 0.56-4.92%. The DLLME technique showed great potential as a sample preparation technique with the recovery percentage of $\leq$ 104.40%. The pesticide residues were not detected in six honey samples collecting from Phayao Province, Thailand. Therefore, this proposed method is suitable for determination of pesticide residues in honey samples.

Keywords: Dispersive liquid-liquid microextraction (DLLME), Gas chromatography (GC), Electron capture detector (ECD), Organochlorine pesticides, Honey

Introduction

In the northern part of Thailand especially in Phayao Province, there are many business bee farms for honey production. Honey is a natural product manufactured by various kinds of bees harvesting nectar from flowers and plants (Cuevas-Glory, Pino, Santiago, & Sauri-Duch, 2007). Honey contains a number of agents believed to be beneficial to human health including antioxidants and essential nutrients. Honey is used as an antibacterial agent. Many areas use pesticides on agricultural products which are a potential toxic contaminant of honey and honeycomb (Kujawski, Pinteaux & Namiesnik, 2012).

Organochlorines such as lindane and dieldrin have been widely used as an insecticide in various areas for agricultural purposes. These substances are very harmful, carcinogenic and take long period to degrade (Jayaraj, Megha, & Sreedev, 2016). The residue pesticides contaminated in honey can be an indicator of environmental pollution (Lozano et al., (2019). Additionally, the monitoring of pesticides concentrations in honey may be required by law or useful information for consumers. The main analytical techniques for determination of organochlorine pesticides in samples are liquid or gas chromatography. Gas chromatographic technique (GC) with electron-capture detector (ECD) is a very useful assay that is selective and sensitive. Moreover, this technique has previously been employed for the determination of organochlorines in honey samples (Rial-Otero,

Gaspar, Moura, & Capelo, 2007). Honey contains fructose, glucose, carbohydrate and some phenolic compounds. These complexing matrices can interfere an analytical process. Therefore, sample pre-treatment methods are necessary for clean-up and preconcentrating samples before instrumental determination (Zacharis, Rotsias, Zachariadis, & Zotos, 2012). Various separation techniques have been used to extract pesticides from honey samples such as liquid-liquid extraction (LLE) (Rodrigues et al., 2018), solid-phase microextraction (SPE) (Rodrigues, 2018; Yang et al., 2011), hollow fiber liquid-phase microextraction (HF-LPME) (Sun et al., 2011), headspace solid phase microextraction (HS-SPME) (Bianchi, Mangia, Mattarozzi, & Musci, 2011; Filho, Santos, & Pereira, 2010). However, some disadvantages of LLE and SPME technique are also reported. Disadvantages of LLE are a long times extraction and a large volume of solvent consumption (mL) which could potentially impose to healthy (Paulino de PinhoAntônio, Neves, Ribeiro de Queiroz, & Silvério, 2010). SPME exhibits instability and swelling in organic solvents (Nerín, Salafranca, Aznar, & Batlle, 2009). Moreover, fiber used in SPME is fragile and sample carry over is found in SPME technique (Kin, & Huat, 2009). Dispersive liquid-liquid microextraction (DLLME) technique was first developed in 2006 by Rezaee and co-workers (Rezaee et al., 2006) using ternary component solvent system including extraction solvent, dispersive or disperser solvent and aqueous solution. This technique can overcome disadvantages of above preconcentration methods. The DLLME procedure was shown in Figure 1. This simple and rapid technique has been used to extract organochlorine pesticides residues in honey. (Zacharis, Rotsias, Zachariadis, & Zotos, 2012; Almeida, Fernandes, & Cunha, 2012)., carbamate pesticides in apples (Zhang et al., 2010) and pesticides from various chemical groups in water samples (Tankiewicz, & Biziuk, 2018). This work aimed to develop a simple and practical method for the determination of lindane and dieldrin in honey samples using DLLME and gas chromatography – electron capture detector (GC-ECD).

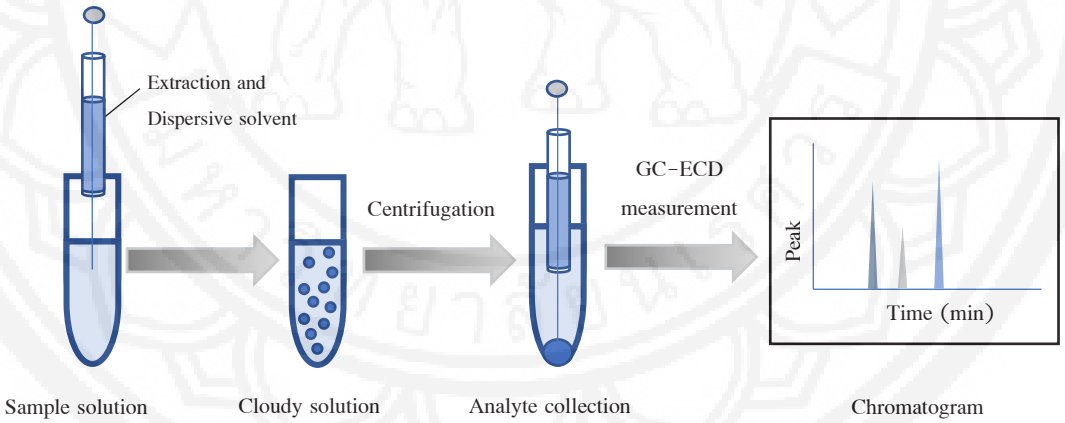


Figure 1 The DLLME procedure

Materials and Methods

Instrumentation and reagents

Gas chromatograph equipped with an electron capture detector, model CP-3800 Prostar, Varian, USA. A Capillary column; FactorFourTM VF-5ms fused silica, Varian, USA. The injection was operated in a splitless/split mode with split ratio of 50:50. All reagents were analytical grade from Merck, Germany. Mixed-pesticide solutions were prepared in n-hexane. Six honey samples were collected from Phayao Province, Thailand.

Effect of extraction solvent volume on DLLME procedure

The selection of an appropriate extraction solvent is crucial for DLLME process. The optimal extractive solvent was carried out with some requirements as follows: higher or lower density than water, a low solubility in water and should form a stable two-phase system in the presence of a dispersive solvent when injected to an aqueous solution. On the basis of these considerations, toluene and dichloromethane were investigated. The influence of the extraction solvent volume on the performance of DLLME procedure, different volume of toluene and/or dichloromethane (300, 400, 500 and 600 μL) with a constant volume of the dispersive solvent acetronitrile (1000 μL) were investigated. Other DLLME parameters such as a mixing time by vortex, centrifugation speed and centrifugation time were constant as following; mixed by vortex for 1 min, centrifuged for 3 min at 2500 rpm. Therefore, the proposed DLLME procedure was caried out lower than 5 min.

The honey samples were weighted (2.000 g) and placed in a beaker. 50 mL of deionized water was added to the beaker and mixed, then the resultant solution placed into a volumetric flask (100 mL) and adjusted by deionized up to volumetric scale. The sample solution (5 mL) was transferred into a centrifuge tube and suitable volume of extraction solvent (toluene or dichloromethane) and 1000 μL of acetronitrile (dispersive solvent) were added. The solution was mixed using vortex for 1 min and then centrifuged for 3 min at 2500 rpm. The upper layer was pipetted and filtered through nylon syringe filter (pore size 0.45 μm) to 2 mL vial bottle. The lindane and dieldrin concentrations in the extracted sample were measured by GC- ECD using the operational condition as shown in Table 1 and the column temperature program was 140 °C for 4 min, 140 to 200 °C with 60 °C/min and 200 to 240 with 50 °C/min (2 min). The concentration of analytes was calculated using the calibration curve that was plotted between peak area (y-axis) and concentration of pesticide standard solution (x-axis).

Effect of column temperature increment rate

The column temperature was programmed as follows: 140 °C for 4 min, 140 to 200 °C and 200 to 240 with 50 °C/min (2 min). The column temperature increment rate (°C/min) in the range of 140 to 240 °C for lindane and dieldrin measurement was investigated from 20 to 80 °C/min and the detector temperature was 330 °C at different gas flows (2.0 to 5.0 mL/min).

Table 1 Operational and optimized conditions for GC-ECD

Parameter	Operational condition
Carrier gas:	Nitrogen (N_2)
Gas flow rate, mL/min:	-
Capillary column:	FactorFourTM VF-5ms fused silica (30 m x 0.25 m i.d., df = 0.2 μm), %phenyl, 5%dimethylpoly siloxane, Varian, USA
Column temperature, °C:	140 °C for 4 min, 140 to 200 °C and 200 to 240 with 50 °C/min (2 min)
Injection;	
Split ratio:	50
Temperature:	250
Volume, μL:	1
ECD detector;	
Temperature:	330
End time, min:	6

Method validation studies

Linearity

A range of organochlorine, lindane and dieldrin standard mixture stock solutions containing 50 - 100 ppm were prepared in n-hexane and stored at 4 °C. Different concentration levels of stock solution were employed due to their sensitivity to the ECD detector. Working standard solutions of a mixture of pesticides (0, 0.05, 0.5, 1, 5, 10, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000 and 10000 ppb) were freshy prepared daily by volume dilution in n-hexane. The solutions were filtered through nylon syringe filter (pore size 0.45 μm) to 2 mL vial bottle. Each bottle was measured by GC-ECD for three replicates. The graph was plotted between peak area (y-axis) and concentration of pesticide standard solution (x-axis).

Repeatability

The repeatability was studied as following. A range of organochlorine, lindane and dieldrin standard mixture stock solutions containing 50 -100 ppm were prepared in n-hexane and stored at 4 °C. Different concentration levels of stock solution were employed due to their sensitivity to the ECD detector. Working standard solutions of a mixture of pesticides at lower concentration (5 ppb) and higher concentration (100 ppb) were freshy prepared daily by volume dilution in n-hexane. The solutions were filtered through nylon syringe filter (pore size 0.45 μm) to 2 mL vial bottle. Each bottle was measured by GC-ECD for three replicates. The relative standard deviation (%RSD) was calculated.

Limit of detection and limit of quantification

LOD and LOQ were investigated by measuring the reagent blank for seven replicates. The values of LOD and LOQ were calculated according to the formula: LOD = 3SD/slope (SD = standard deviation) and LOQ = 10SD/slope, respectively.

Recovery

Recovery study was investigated by preparation six sets of sample solutions. For each set, the sample solutions were prepared by spiking the different concentrations (0, 5, 7 and 10 ppb) of the organochlorine, lindane and dieldrin standard mixture stock solutions to each beaker containing 2 g of honey sample collected from Mae Chai. The solutions were mixed and transferred to volumetric flasks. The solutions were extracted using the DLLME technique. The other sets of sample solutions, collected from Chiang Kham, Chiang Muan, Phu Kamyao, Mueang and Pong were also prepared by the procedure as described above. The prepared sample solutions were measured by GC-ECD under the optimum condition for three replicates. Recovery data was calculated by integrating the peak area of the plot between concentration of the mixed pesticide standard solution (x-axis) and peak area (y-axis).

Determination of Lindane and Dieldrin with and without preconcentration by DLLME

To investigate the efficiency of DLLME procedure, concentration of lindane and dieldrin in honey sample collected from Pong with and without preconcentration by DLLME was measured by GC-ECD under the optimum condition for three replicates.

Determination of Lindane and Dieldrin in real honey samples

Concentration of lindane and dieldrin in six honey samples with preconcentration by DLLME was investigated by GC-ECD under the optimum condition for three replicates.

Results and Discussion

The effect of extraction solvent volume on extraction efficiency

Disperser solvent is a key for helping the extraction solvent forming fine droplets in aqueous samples (Kabir, Locatelli, & Ulusoy, 2017), therefore in this work acetronitrile was selected as a disperser solvent with fixed volume of 1,000 μL. The effect of volume of extraction solvent was studied using toluene comparing with dichloromethane ranging the volume from 300 to 600 μL. Increasing ratios of extraction solvent with dispersive solvent of 300:1000, 400:1000, 500:1000 and 600:1000 μL were investigated. The results as shown in Table 2 revealed that the ratio of extraction solvent with dispersive solvent of 300:1000 (toluene: acetonitrile) achieved a good recovery value (more than 81%) while dichloromethane gave the lower recovery (<56%) for both lindane and dieldrin. This can be explained as follows. Volumes of extraction solvents affected the enrichment factor when increasing the solvent volumes, the volumes of solvent droplets obtained after centrifugation increases, resulting in a decrease in the concentration of the extracted substances, thus the enrichment factor decreased. This result was in accordance with the result that reported by Kim and co-worker which toluene (350 μL) exhibited the highest efficiency compared with n-hexane and isooctane (Kim & Huat, 2009). Therefore, the ratio of extraction solvent (Toluene) with dispersive solvent (Acetonitrile) of 300:1000 μL was selected for further experiment and the optimal condition for DLLME procedure can be summarize as shown in Table 3.

Table 2 Type and volume of extraction solvent on extraction of lindane and dieldrin in honey samples

Analyte	Volume of extraction solvent (μL)	%Recovery (n=3)	
		Toluene	Dichloromethane
Lindane	300	88.46	55.62
	400	75.30	74.42
	500	61.98	50.74
	600	50.35	47.63
Dieldrin	300	81.15	54.86
	400	54.50	48.49
	500	42.20	33.92
	600	32.55	32.06

Table 3 Optimum condition for DLLME procedure

Parameter	DLLME condition
Extraction speed	2500 rpm
Centrifugation time	3 min
Extraction solvent, volume	Toluene, 300 μL
Dispersive solvent, volume	Acetonitrile, 1000 μL

Effect of column temperature increment rate on retention time

The effect of temperature increment rate at various gas flow rates on analytical column as shown in Figure 2 revealed that at lower temperature increment rate (20 °C/min) and flowrate (2 mL/min) achieved longer retention time (~5.4 min). On the other hand, increasing temperature increment rate and gas flowrates decreased in the retention time (~2.9 min). The column temperature increment rate in the range of 60 - 80 °C/min and gas flow rate in the range of 3-5 mL/min revealed a stable retention time. However, this condition was not fit for a complex substance. Therefore, the column temperature increment rate of 60 °C/min and gas flow rate of 3 mL/min were selected for further experiment. And the optimum condition for GC-ECD analysis can be summarize as shown in Table 4.

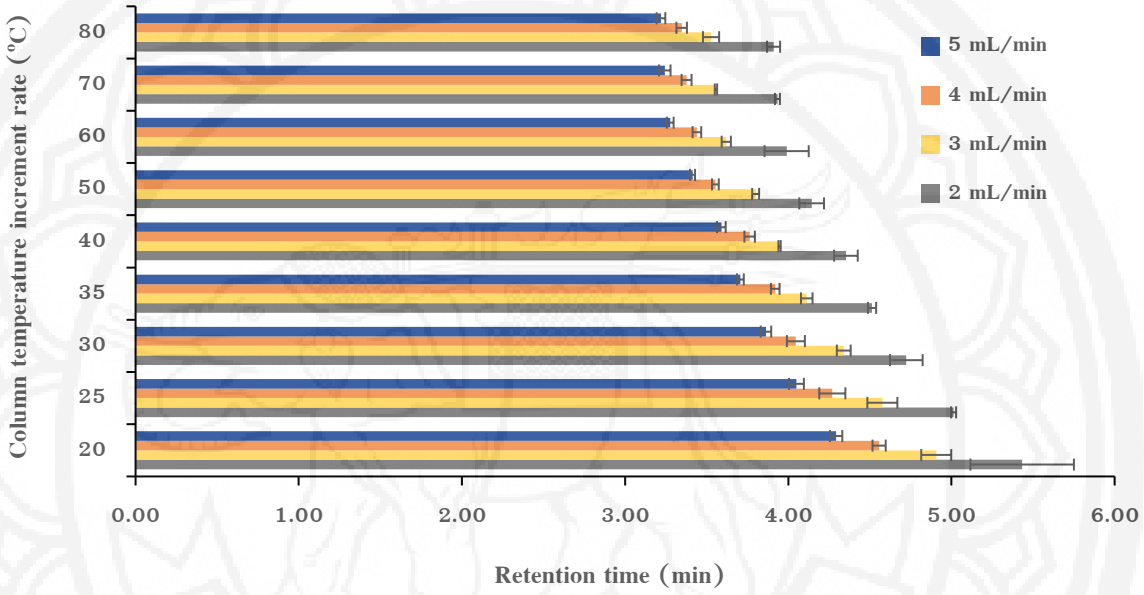


Figure 2 Effect of column temperature increment rate at various flowrates (n=3)

Table 4 Optimum condition for GC-ECD analysis

	GC-ECD condition
Carrier gas:	Nitrogen
Gas flow rate:	3 mL/min
Capillary column:	FactorFourTM VF-5ms fused silica (30 m x 0.25 m i.d., df = 0.2 μm), %phenyl, 5%dimethylpoly siloxane, Varian, USA
Column temperature:	140 °C for 4 min, 140 to 200 °C with 60 °C/min and 200 to 240 with 50 °C/min (2 min)
Injection;	
Split ratio:	50
Temperature:	250 °C
Volume:	1 μL
ECD detector;	
Temperature:	330 °C
End time:	6 min

Method validation studies

The validate method such as linearity, repeatability, reproducibility, LOD, LOQ and %Recovery were studied using GC-ECD condition as in Table 4 and the column temperature program was 140 °C for 4 min, 140 to 200 °C with 60 °C/min and 200 to 240 with 50 °C/min (2 min). All results were shown as following.

Linearity

The linearities of the lindane and dieldrin standard solutions were studies, in the range of 0 to 10000 ppb (n= 3). The results as shown in Figure 3 revealed that the linearity found to be in the range between 0.05-1000 ppb with the linear regression more than 0.999 for lindane (Figure 3) and found to be 1-3000 ppb with the linear regression more than 0.997 for dieldrin (Figure 4). However, the concentrations of lindane and dieldrin were calculated using the graph that was plotted between peak area (y- axis) and concentration of the mixed standard lindane and dieldrin in the range of 0 to 100 ppb (x-axis) and Peak area.

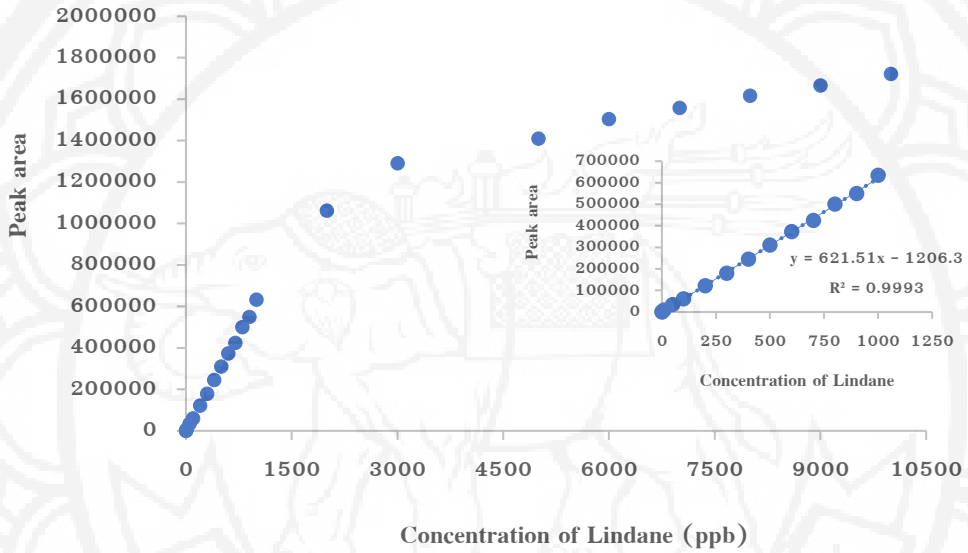


Figure 3 Graph of the plot between concentration of lindane (ppb) and peak area

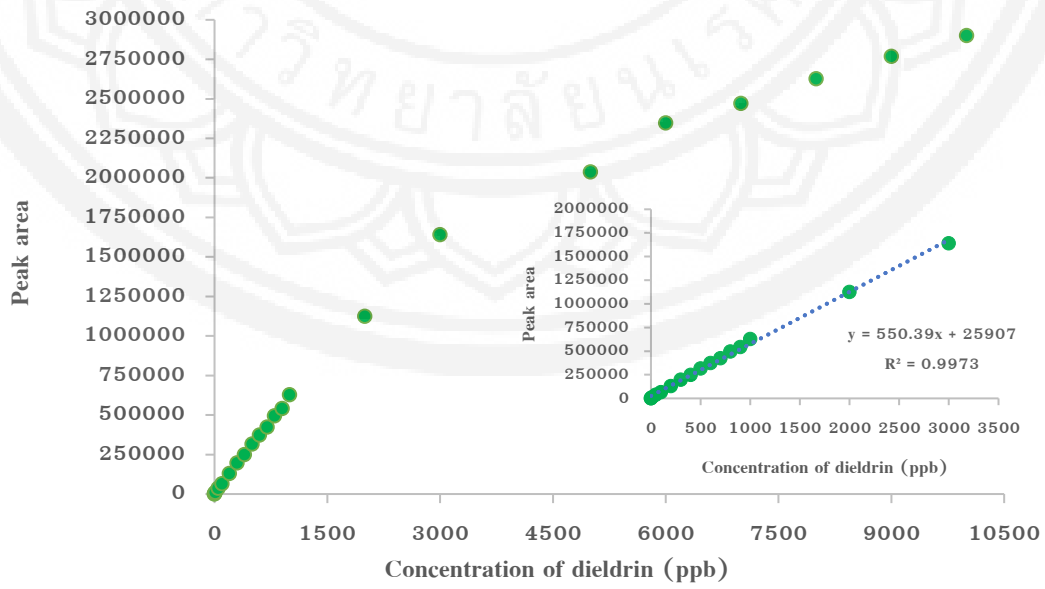


Figure 4 Graph of the plot between concentration of dieldrin (ppb) and peak area

Repeatability

The results shown in Table 5 revealed that the DLLME equipped with GC-ECD showed good precision. The repeatability values for lindane and dieldrin at lower concentration of mixed standard (5 ppb mixed lindane and dieldrin) were 4.59 and 4.92%, respectively. At higher concentration (100 ppb mixed lindane and dieldrin), the repeatability values (n=3) for lindane and dieldrin were 0.56 and 1.32%, respectively.

Table 5 Repeatability data for the lindane and dieldrin determination

Repeatability data					
Time	Peak area of Lindane		Peak area of Dieldrin		Bottle
	5 ppb	100 ppb	5 ppb	100 ppb	
1	4360	79648	5266	83902	1
2	4429	79810	5045	83882	2
3	4361	78785	5050	80751	3
4	4088	79845	4878	83387	4
5	4275	79103	5044	83203	5
6	4420	80021	5327	83480	6
7	3905	79525	4584	83612	7
Mean	4263	79534	5028	83174	Mean
S.D.	196	442	247	1098	S.D.
%RSD	4.59	0.56	4.92	1.32	%RSD

Limit of detection and limit of quantification

LOD and LOQ values were calculated by measuring the blank for 7 times. The results showed that the LOD and LOQ for lindane were 0.18 and 0.60 ppb, respectively and for dieldrin were 0.27 and 0.87 ppb, respectively.

Recovery study

Recoveries were calculated as the percent ratio between the found and the known concentrations. The recovery results were shown in Table 6. The DLLME procedure for preconcentration of honey samples (measured for 3 times) was accurate with the recovery values in the range of 61.31-104.40%.

All validated results demonstrated that the accuracy and precision of the proposed method were obtained when DLLME was used. Therefore, the approved preconcentration procedure was valid to investigate the amount of lindane and dieldrin in real samples.

Table 6 Recovery values of the preconcentration step for determination of lindane in honey samples

Sample name	Spiked lindane and dieldrin (ppb)	Concentration of lindane ± S.D., (ppb), n = 3	%Recovery	Concentration of dieldrin ± S.D., (ppb), n = 3	%Recovery
Mae Chai	0	0	-	0	-
	5	5.182±0.025	103.64	3.933±0.048	78.66
	7	6.027±0.032	86.10	4.823±0.046	68.90
	10	8.288±0.098	82.88	6.573±0.038	65.73
Chiang Kham	0	0	-	0	-
	5	4.467±0.032	89.34	3.300±0.058	66.00
	7	6.489±0.022	92.70	5.327±0.087	76.10
	10	8.116±0.171	81.16	6.131±0.082	61.31
Chiang Muan	0	0	-	0	-
	5	4.617±0.022	92.34	3.667±0.111	73.34
	7	7.043±0.231	100.61	6.764±0.086	96.63
	10	8.597±0.112	85.97	6.226±0.092	62.26
Phu Kamyao	0	0	-	0	-
	5	4.266±0.147	85.32	3.394±0.167	67.88
	7	6.508±0.023	92.97	6.238±0.042	89.11
	10	9.531±0.068	95.31	7.096±0.102	70.96
Mueang	0	0	-	0	-
	5	4.938±0.035	98.76	3.855±0.098	77.10
	7	6.880±0.087	98.29	7.308±0.124	104.40
	10	9.620±0.078	96.20	7.600±0.044	76.00
Pong	0	0	-	0	-
	5	4.694±0.050	93.88	3.395±0.114	67.90
	7	6.301±0.041	90.01	6.062±0.093	86.60
	10	9.699±0.102	96.99	7.220±0.060	72.20

Determination of Lindane and Dieldrin with and without preconcentration by DLLME

The GC chromatogram of honey sample from Pong district without preparation by DLLME was shown Figure 5. The chromatogram of honey samples without preparation revealed many impurity peaks. Figure 6 showed a very clear chromatogram with the retention time at 3.6 min for lindane (spiked 5 ppb lindane standard) and retention time at 4.9 min for dieldrin (spiked 5 ppb dieldrin standard). Therefore, the DLLME technique was a potential preparation technique for extraction of lindane and dieldrin from complex substances like honey samples.

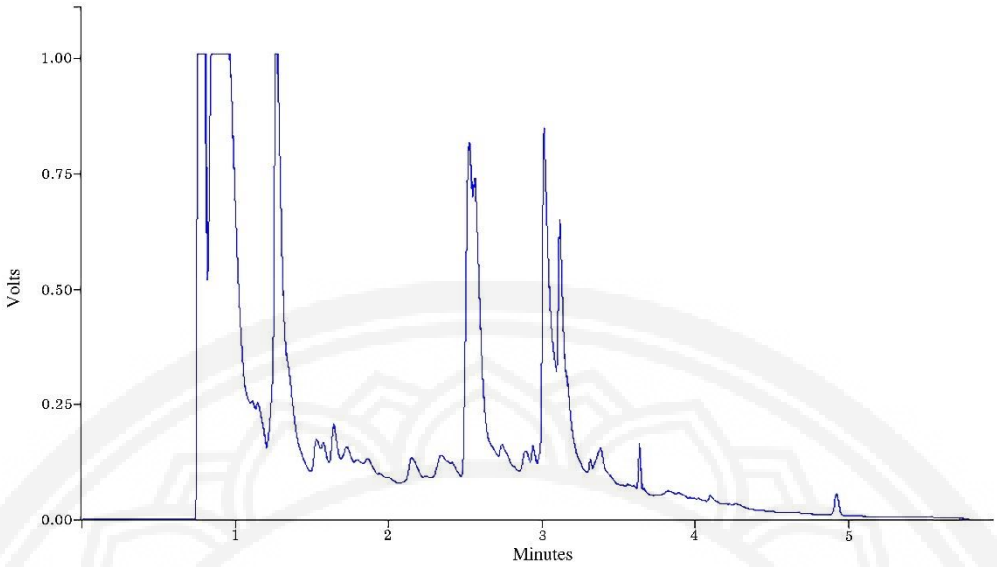


Figure 5 GC chromatogram of honey samples from Pong District without preparation by DLLME technique

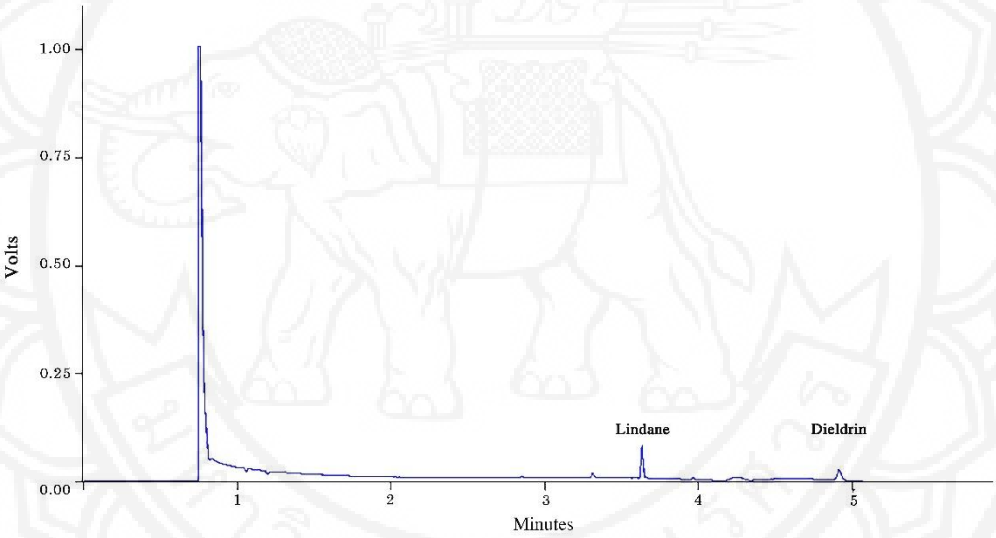


Figure 6 GC chromatogram of honey samples from Pong District with preparation by DLLME technique(Spiked with 5 ppb lindane and 5 ppb dieldrin standard)

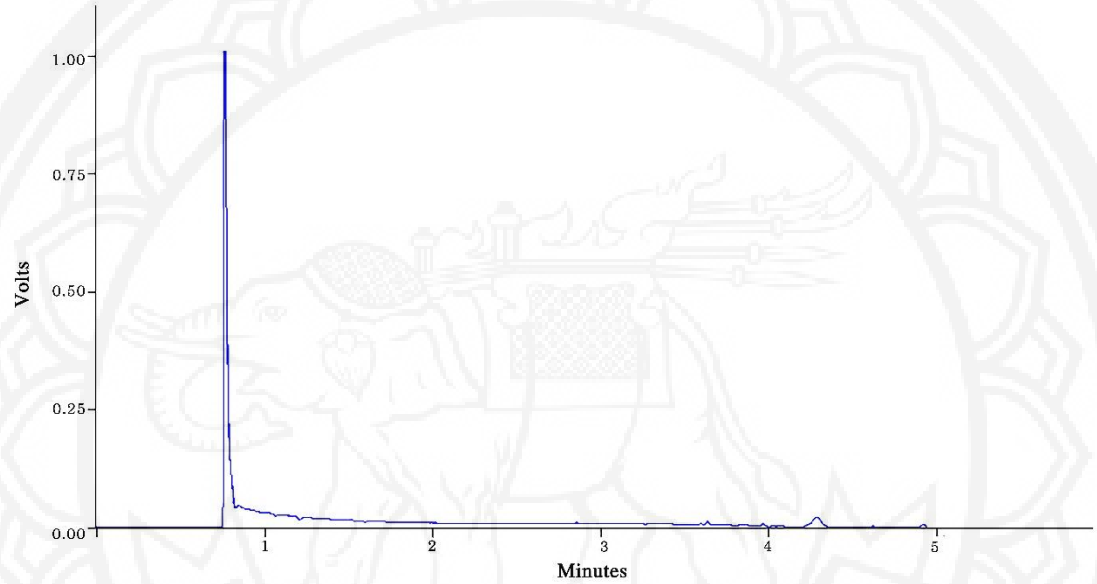
Determination of Lindane and Dieldrin in real honey samples

Concentrations of lindane and dieldrin in honey samples collected from 6 areas were investigated using the DLLME technique for sample preparation step and consequently measured by GC-ECD with optimum condition shown in Table 3 and 4. After six honey samples were prepared by DLLME with optimum condition, the concentrations of lindane and dieldrin were measured for 3 times. The lindane and dieldrin contents were calculated by integrating the peak area of the plot between concentration of the mixed pesticide standard solution (x-axis) and peak area (y-axis). The results of peak areas were shown in Table 7 and some chromatograms of honey samples collected from Chiang Kham and Phu Kamyao District were shown in Figure 7 (a-b). Based on the LOQ values for lindane (0.60 ppb) and dieldrin (0.87 ppb), the results revealed that both lindane and dieldrin were not detected in all six honey samples.

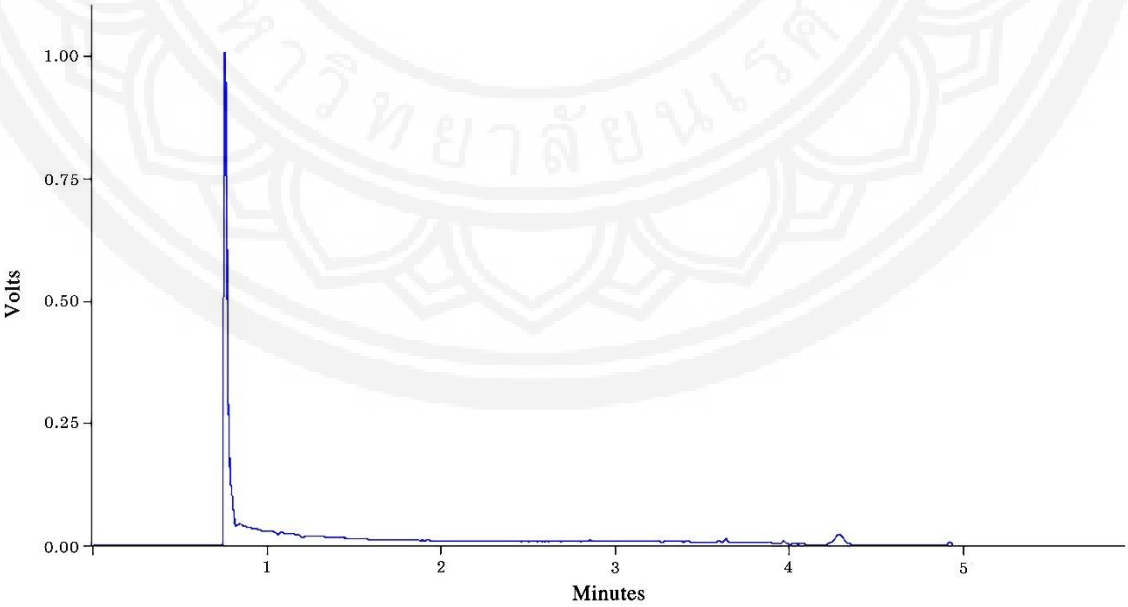
Table 7 Concentrations of lindane and dieldrin in six honey samples collected from various areas in Phayao Province

Sample name	Average Peak area (n=3)		Concentration (ppb)	
	Lindane	Dieldrin	Lindane	Dieldrin
Mae Chai	2369 ± 48	4973 ± 178	ND	ND
Chiang Kham	2460 ± 57	3580 ± 65	ND	ND
Chiang Muan	1831 ± 45	2607 ± 65	ND	ND
Phu Kamyao	1643 ± 39	8040 ± 552	ND	ND
Mueang	1657 ± 60	3582 ± 35	ND	ND
Pong	3200 ± 87	8670 ± 217	ND	ND

*ND = Not detected



(a)



(b)

Figure 7 Chromatograms of honey samples from (a) Chiang Kham and (b) Phu Kamyao District

Conclusion

In this study, dispersive liquid-liquid microextraction (DLLME) was used to extract organochlorines, lindane and dieldrin from honey samples prior to determination by gas chromatography-electron capture detector (GC-ECD). The complexing matrices could be removed from honey samples by DLLME methods and the results of GC-ECD validation presented good precision and accuracy with low detection limits at ppb level for lindane and dieldrin determination. This preliminary method could be used to determine lindane and dieldrin in honey samples from many areas of Phayao Province. Future work, some parameters such as disperser solvent type, effect of ionic strength, extraction time and centrifugation speed would be focused to optimize the extraction procedure.

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