# DEVELOPMENT OF METHOD FOR SIMULTANEOUS DETECTION OF DIBUTYL PHTHALATE AND OLEAMIDE IN STINGLESS BEE HONEY HARVESTED FROM PLASTIC CUPS BY SOLVENT TERMINATED DISPERSIVE LIQUID-LIQUID MICROEXTRACTION AND HPLC

BY

KOO YEE PING

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# LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

| | |
|---|---|
| DBP | Dibutyl Phthalate |
| HPLC | High Performance Liquid Chromatography |
| LOD | Limit of Detection |
| LOQ | Limit of Quantitation |
| ND | Not Detected |
| $R^2$ | Coefficient of Determination |
| SML | Specific Migration Limit |
| S/N | Signal-to-Noise Ratio |
| ST-DLLME | Solvent Terminated Dispersive Liquid-Liquid Microextraction |

# ABSTRAK

Permintaan yang meningkat terhadap madu kelulut di Malaysia telah merangsang para penternak lebah untuk meletakkan bekas plastik yang strukturnya menyerupai tempat penyimpanan madu di dalam sarang lebah sebagai satu inisiatif untuk meningkatkan penghasilan madu. Tetapi, tahap asid madu kelulut berkemungkinan menyebabkan migrasi bahan organik dari plastik ke dalam madu. Pendedahan kepada bahan toksik untuk jangka masa yang panjang boleh memudaratkan kesihatan. Tujuan kajian ini adalah untuk menentukan kehadiran dibutyl phthalate (DBP) dan oleamide dalam madu kelulut yang dikumpul dari bekas plastik. Satu kaedah yang menggabungkan teknik *Solvent Terminated Dispersive Liquid-Liquid Microextraction* (ST-DLLME) dan *High Performance liquid Chromatography* (HPLC) untuk mengekstrak dan mengesan kedua-dua bahan ini telah dikenalpasti. Langkah-langkah untuk menentukan parameter optimum ST-DLLME seperti jenis *extraction solvent* dan *disperser solvent* telah dibuat. Parameter optimum HPLC yang digunapakai dalam kajian ini termasuk penggunaan turus Zorbax Eclipse XDB-C18, acetonitrile: methanol (70:30), dan gelombang pengesanan pada 202nm. Keseluruhan proses HPLC hanya mengambil jangkamasa 10 minit sahaja. Julat kelinearan untuk DBP diantara 0.5-50 µg/g; sedangkan julat oleamide diantara 10-250 µg/g. *Limit of detection* untuk DBP adalah 0.15µg/g; manakala untuk oleamide adalah 4µg/g. *Limit of quantitation* untuk DBP dan oleamide adalah masing-masing 0.5µg/g dan 11µg/g. Penilaian terhadap kaedah ini menunjukkan penghasilan semula untuk DBP sebanyak 61.6%, manakala oleamide 74.7%. *Relative standard deviation* untuk DBP dan oleamide menunjukkan 7.3% dan 8.9% masing-masing (n=3). Kaedah ini merupakan teknik yang sensitif

dan juga cepat dalam mengekstrak kedua-dua DBP dan oleamide daripada madu kelulut. Ianya dapat mengesan tahap kepekatan di bawah tahap limitasi yang spesifik seperti yang ditentukan dalam EU Commission 10/2011 untuk produk yang digunakan dalam pengisian makanan. Kedua-dua DBP dan oleamide tidak dikesan dalam madu kelulut yang diperolehi dari bekas plastik dengan menggunakan teknik ini.

# DEVELOPMENT OF METHOD FOR SIMULTANEOUS DETECTION OF DIBUTYL PHTHALATE AND OLEAMIDE IN STINGLESS BEE HONEY HARVESTED FROM PLASTIC CUPS BY SOLVENT TERMINATED DISPERSIVE LIQUID-LIQUID MICROEXTRACTION AND HPLC

## ABSTRACT

Recent increased demand for stingless bee honey in Malaysia have stimulated alternative methods to increase honey yields by using plastic cups as artificial honey pots within the beehives. However, honey's acidity may lead to possible migration of plastic additives, where prolonged consumption may affect body's physiological responses. This study was carried out to determine the presence of dibutyl phthalate (DBP) and oleamide in stingless bee honey collected from plastic cups. A rapid method for simultaneous extraction and detection of both analytes by Solvent Terminated Dispersive Liquid-Liquid Microextraction (ST-DLLME) coupled with High Performance Liquid Chromatography (HPLC) was developed. ST-DLLME parameters such as type of extraction solvent and disperser solvent were optimized. HPLC parameters optimized to produce good compound separation were Zorbax Eclipse XDB-C18 column, mobile phase acetonitrile: methanol (70:30) and detector wavelength 202nm with total

runtime 10 minutes. Good linearity was observed for DBP ranged between 0.5-50 µg/g; and oleamide ranged between 10-250 µg/g. Limits of detection for DBP was 0.15µg/g; while oleamide was 4µg/g. Limits of quantitation for DBP and oleamide were 0.5µg/g and 11µg/g respectively. Spiked recovery for DBP was 61.6%, oleamide was 74.7%. The relative standard deviation for DBP and oleamide were 7.3% and 8.9% respectively (n=3). This developed method presents a sensitive and rapid detection procedure for both compounds, as it is able to detect concentrations below the specific migration limits determined by EU Commission 10/2011 for food contact materials. Both DBP and oleamide were not detected in all the honey samples collected.

# Chapter 1 INTRODUCTION

## 1.1 Background of Study

Honey is widely known for its natural sweetening and medicinal properties (Souza et al. 2006; Pérez-pérez et al. 2013). Traditional and folk medicine have also long practiced consuming honey for general wellbeing (Bogdanov et al. 2008; Kwakman & Zaat 2012). Recent advances have claimed that the presence of polyphenols in honey contribute to its antioxidant capacity; hence reduce the inflammatory responses in the body (Alzahrani et al. 2012). As many non communicable diseases arise from our body's over reactive inflammatory response, honey may benefit in its antioxidant properties in addition to adopting a healthy lifestyle.

Honey is produced when bees have gathered and processed the nectar from nearby plants. There are different tribes present within the family of bees (*Apidae*); namely the honey bees, orchid bees, stingless bees etc. Each species are distinctive in the honey that they produce. The most commonly marketed honeys are those from the honey bees, *Apis mellifera*. However, there is another type of honey; the stingless bee honey that is also widely produced and consumed in the subtropics. It is sold at a much higher price due to its scarcity and its exotic taste compared to the *Apis* honey (Pimentel et al. 2013).

The recent increase in demand of stingless bee honey in Malaysia due to the public's interest and awareness in consuming natural products for general wellbeing have prompted beekeepers to increase their supply to meet the demands. One of the measures taken by the beekeepers to generate a larger amount of honey is by placing plastic cups that mimic the shape and size of the stingless bee's natural honey pots. This initiative of providing the bee colonies with ready-made honey storage pots was to encourage them to spend more time collecting nectar and producing honey. However, the use of plastic cups made of polypropylene pose a

concern due to its prolonged contact with stingless bee honey which are acidic (pH range 3 to 4.5) (Souza et al. 2006). The acidity of honey together with recent elevated environmental temperatures may promote the leaching of organic migrants from plastics into the honey.

Organic migrants of interest in this study are dibutyl phthalate and oleamide. Dibutyl phthalate was chosen instead of others because it is one of the commonly reported phthalates to cause endocrine disruption (Moreira et al. 2013). Slip additive oleamide was selected due to recent reports of leaching from disposable laboratory plasticware made of polypropylene (McDonald et al. 2008).

## 1.2 Literature Review

### 1.2.1 Stingless Bees

Stingless bees, as the name suggests, do not sting, they only bite in defence if threatened. There are more than 500 species of stingless bees mainly thriving in the tropical and subtropical regions known up till date and more species are being identified (Bradbear 2009). Stingless bees normally live in crevices and enclosed spaces like those within the hollow tree trunks; but some also share their hives with ants or termites. Stingless bee nests are distinctive from their honey bee counterparts wherein their nests normally appear in the form of horizontal pots made of cerumen and propolis, for honey and pollen storage. Further within the honey and pollen pots lies the brooding chamber for the larvae to grow. Whereas honey bees nests are built in the form of honey combs hanging from tree branches. The stingless bee honey has a sour taste and appears less viscous due to its higher acidity and water content compared to *Apis* honey (Bijlsma, L., de Bruijn, L.L.M., Martens, E.P and Sommeijer 2006; Pérez-pérez et al. 2013). The many benefits and medicinal properties of stingless bee honey are further discussed below.

### 1.2.2 Wound Healing Effects of Honey

It is mentioned in a review by Manyi-Loh (2011) that the local application of honey can promote wound healing through a few mechanisms. Being a moist substance; it provides sufficient moisture to the damaged skin surface and also encourages debridement of necrotic tissue in order to encourage tissue regrowth. Hydrogen peroxide in honey also possesses antibacterial activity that can prevent wound infection from local skin colonizers (Bogdanov et al. 2008; Manyi-Loh 2011). Hydrogen peroxide and gluconic acid are products of glucose metabolism catalyzed by glucose oxidase (Bogdanov et al. 2008). Glucose oxidase is an enzyme that is secreted by bees into the nectar during honey production (Kwakman & Zaat 2012; Pimentel et al. 2013). The acidic medium provided by honey apart from inhibiting microbial growth, also enhances oxygen supply to damaged tissues by promoting oxygen dissociation from oxyhaemoglobin (Rendl et al. 2001; Simon et al. 2009). In addition, the acidic pH also prevents ammonia, a byproduct of bacterial protein metabolism from producing toxic effects and malodour from the wound; as an alkaline environment is not favourable for wound healing (Simon et al. 2009). Honey from stingless bees was also shown to have *in-vitro* activity against gram positive *Staph. aureus* and gram negative organism *E. coli* (Pimentel et al. 2013).

### 1.2.3 Antioxidant Effects of Honey

Polyphenols are the minor constituents thought to contribute to the antioxidant properties of honey (Alzahrani et al. 2012). Polyphenols are secondary plant metabolites which include the phenolic acids and flavonoids (Pérez-pérez et al. 2013). This effect was studied by Silva et al. (2013) on samples of Melipona honey collected from Brazil, where the results showed that the total phenolic content correlated with the antioxidant capacity of honey. However, bees produce honey with varying levels and types of antioxidants (Pérez-pérez et al. 2013). This is very much dependent on the bee species as different species will favour nectar

from different botanical origins (Bogdanov et al. 2008; Pimentel et al. 2013). Moreover, the distribution of floral species also varies with geographical location and weather conditions (Silva et al. 2013).

As one colony of stingless bee can only produce approximately 200 grams to 5 kilograms of honey per year (Bradbear 2009), it is difficult to sustain the demand with only this meagre production. Moreover, increasing deforestation activities have also substantially reduced the wild colonies of stingless bees (Oliveira et al. 2013). Hence beekeepers have brought beekeeping from the wild into their own farms. Log hives containing bees nest are obtained from the forest and placed on wooden boxes. The colony will then expand their honey and pollen pots within the wooden boxes. This ensures that the honey can be harvested without damaging the nest, and also the sustained production from the colony. Some beekeepers in Malaysia came up with an additional initiative by placing plastic cups lined in rows within the wooden boxes that mimic the shape and arrangement of the natural honey and pollen pots. Their aim was to reduce the time required by the stingless bees to make the honey pots and thus the bees can spend more time collecting nectar to increase honey production. Figure 1-1 shows the arrangement of honey pots within the beehives.

**Figure 1-1**: Stingless bee hive with plastic cups for honey and pollen storage.

### 1.2.4 Plastic Cups as Artificial Honey Pots

Though plastic cups can be a convenient method to increase honey yield, nonetheless there are concerns regarding plastic additives that may leach into the honey that comes into contact with it. Plastics are polymeric materials made from petrochemicals widely used for storage and transportation purposes. Its light weight and relatively cheaper cost compared to other bulky materials such as glass makes it favourable among consumers. However, many additives have been added into plastics to improve its performance. These range from plasticizers for improved flexibility such as the phthalate compounds; slip agents such as the fatty acid amides to facilitate extrusion of plastics from their moulds during manufacturing; to

antioxidants used for the resistance against oxidative degradation processes and many more (Bhunia et al. 2013).

A number of factors can affect the migration of these plastic additives into food. The nature of food matrix was one of the effects demonstrated by Xu et al. (2010), where eight phthalic acid ester compounds were found to be present in larger amounts in cooking oil (fatty food) than in mineral water (aqueous food). Temperature can also be another contributing factor. Higher temperatures may affect the additive's solubility between the polymer and food phase at equilibrium hence affecting its partition coefficient (Bhunia et al. 2013). Prolonged duration of plastic use is also associated with increased additive migration as observed by larger amounts of Bisphenol A (BPA) migration from polycarbonate infant feeding bottles that were used a hundred times compared to brand new ones (Nam et al. 2010). Plastic aging often occurs with repeated use resulting in the change of plastic structure and thus increased migration of plastic additives into food.

Stingless bee honey is acidic in nature with pH that ranges from 3 to 4.5 (Souza et al. 2006). There is concern about the leaching of plastic additives from the artificial honey pots due to honey's acidity. The effect of pH on phthalate migration was demonstrated when phthalates were found to leach from polyethylene terephthalate bottles containing soft drinks at acidic pH ranging between 2.75-2.82 (Bošnir et al. 2007). Moreover, stingless bee honey also has high moisture content, which can potentially attract additives like phthalates from plastics, as phthalates are not covalently bonded to the polymer structure (Moreira et al. 2013).

### 1.2.5 Phthalates

Phthalates, or otherwise known as esters of phthalic acids are plasticizers used to impart strength and flexibility to plastics (Bhunia et al. 2013; Mousa et al. 2013). A wide variety of plastics such as polyolefins and polyvinyl chloride (PVC), utilize additives such as dibutyl phthalate (DBP), benzylbutyl phthalate (BBP), di-ethylhexyl phthalate (DEHP) and many more.

They are contents in consumer products like toys, cosmetics, building materials and food packaging (Whyatt et al. 2014). Phthalates are known to be endocrine disruptor compounds in animal studies; these exogenous sources of chemicals on prolonged exposure may disturb the body's endocrine system and also affect their offspring (Moreira et al. 2013). Phthalates have the ability to cross the placenta and breast milk to the offspring in animal studies due to its lipophilicity (U.S. EPA 2007). Hormones that are reportedly disrupted by phthalates are the estrogens, thyroid, androgens, and the peroxisome proliferator activated receptor (PPAR) pathway (Tucker 2015).

According to the United States Environment Protection Agency (US EPA) Toxicity and Exposure Assessment for Children's Health Chemical Summary on Phthalates 2007; animal studies involving rats showed that the increased incidence of developmental disorders like cleft palate and skeletal malformation are associated with phthalate exposure. In addition, reproductive tract disorders such as reduced anogenital distance and undescended testes in newborn male rats are also linked to prenatal exposure of dibutyl phthalate. However, evidence of teratogenicity due to phthalates was not limited to animal studies only. A multicentre cohort study which recruited pregnant women from prenatal clinics across Los Angeles, Minneapolis and Columbia reported that the reduction in anogenital distance in boys aged 2 to 36 months were significantly associated with their mother's prenatal urinary phthalate metabolite concentrations (Swan et al. 2005). The phthalate metabolites that had an inverse relationship with the anogenital index (AGI); which is the ratio of anogenital distance to the body weight on examination (mm/kg) include monoethyl phthalate (MEP), monobutyl pththalate (MBP) and mono isobutyl phthalate (MiBP). With the effects of reproductive and developmental toxicity on humans, the Endocrine Society Annual Meeting 2015 recently held in San Diego reported that human exposure to phthalates was associated with an extra 618,000 cases of infertility treatments, bringing the cost to about €4.71 billion annually (Hauser et al. 2015). An additional 24,280 deaths of men aged between 55-64 years old in the European Union were attributed to the cardiovascular events related to the reduction in testosterone level which is thought to be associated with phthalate exposure (Hauser et al. 2015).

Toxicological studies have also shown that obesity and diabetes is another endocrine disorder that can be induced by phthalate exposure. A study carried out on a population of 70 year old women in Sweden looked at the relationship of several obesity markers with serum phthalate concentrations; the increase in waist circumference and abdominal subcutaneous fat was shown to be positively related to serum mono-isobutyl phthalate (MiBP), a metabolite of dibutyl phthalate (Lind et al. 2012). A cross sectional study also demonstrated a positive association between the urinary phthalate metabolites and diabetes among American women aged 20-79 years old (James-Todd et al. 2012). Phthalates have been identified as agonists to the peroxisome proliferator activated receptor (Desvergne et al. 2009). This is a family of nuclear transcription factors which are responsible in insulin sensitization, lipid storage and metabolism in the body (Desvergne et al. 2009; Lind et al. 2012). Apart from the high calorie intake and sedentary lifestyle which are known causes that contribute to obesity and diabetes; activation of PPAR by phthalates may pose as an additional factor to the increased prevalence worldwide (Lind et al. 2012; James-Todd et al. 2012).

### 1.2.6 Oleamide

Another organic compound associated with plastics made of polypropylene is oleamide. Oleamide is chemically known as 9-octadecanamide, a fatty acid amide slip agent added into plastics to facilitate extrusion during manufacture (Bhunia et al. 2013). It is also an endogenous sleep inducing chemical in the cerebrospinal fluid of the brain; first discovered and isolated from sleep deprived cats (Leggett et al. 2004; Solomonia et al. 2008). Oleamide is associated with the slow wave phase of the sleep cycle (Leggett et al. 2004). In vivo studies have shown that exogenous oleamide administration to mice and rats induce hypothermia and reduce locomotion (Huitrón-Reséndiz et al. 2001). Catalepsy, a state of prolonged and unusual rigid fixed body posture was also observed in mice administered with oleamide (Leggett et al. 2004). In a study done by Murillo-Rodríguez et al. (2001), oleamide at doses of 30-50mg/kg facilitated

memory loss with no apparent memory recall after 24 hours with more prominent effects seen at higher doses. In addition, oleamide also induced a significant increase in food intake in Wistar rats (Martínez-González et al. 2004). These effects were thought to be associated with its binding activity as an agonist to the cannabinoid receptor CB1 (Leggett et al. 2004; Martínez-González et al. 2004).

The recent reports of oleamide leaching from disposable laboratory plasticware made of polypropylene (Olivieri et al. 2012) presents a concern as the public may be exposed from food contact materials through ingestion. Chronic exposure may affect our body's normal physiological processes.

### 1.2.7 Solvent Terminated Dispersive Liquid-Liquid Microextraction

Sample pre-treatment and pre-concentration is an important step prior to sample analysis. It serves to remove contaminants or matrix that can possibly interfere with the target analytes. In addition, analytes of interest can also be sufficiently concentrated for it to be detectable by the instrument. In this study, the method selected that can serve these purposes is an extraction method termed solvent terminated dispersive liquid-liquid microextraction (ST-DLLME).

DLLME is a new extraction technique developed for sample pre-concentration or analyte enrichment prior to sample analysis these recent years. Before the development of microextraction techniques, more time consuming methods like liquid-liquid extraction and solid phase extraction were more widely used. However, due to the recent growing awareness to reduce hazardous wastes generated from experimental work, it has triggered the development of methods that use less organic solvents. The use of markedly low volumes of organic solvents in DLLME together with its rapid extraction technique makes it comparatively a more favourable method of extraction to its conventional counterpart liquid-liquid extraction (Guo & Lee 2011; Lv et al. 2013) .

## 1.3 Aim of the Study

The aforementioned issues bring about the aim of this study, which was to measure organic migrants in the commercially produced stingless bee honey. The honeys used for analysis were harvested from plastic cups, which acted as artificial honey pots for honey storage.

## 1.4 Objectives

1. To measure DBP and oleamide in honey samples, obtained from the farm that use plastic cups as artificial honey pots.
2. To develop a simple extraction method for organic compounds (DBP and oleamide) using solvent terminated dispersive liquid-liquid microextraction.
3. To develop a sensitive and reliable measurement for DBP and oleamide using High Performance Liquid Chromatography.

# Chapter 2 METHOD

## 2.1 Honey Samples

Honey samples from artificial honey pots (plastic cups) were collected from Syamille Agrofarm, Kuala Kangsar. Blank honey collected from naturally made stingless bee honey pots acted as positive and negative controls. Control honey samples were spiked with known concentrations of DBP and oleamide in the optimization of extraction conditions. Collected honey were stored in amber glass bottles and kept at 4$^{o}$C until further use.

## 2.2 Reagents and Standards

Standards dibutyl phthalate 99% and oleamide analytical standard were both purchased from Sigma Aldrich. Mobile phase solvents acetonitrile and methanol were both HPLC grade (QRec). Other analytical grade reagents such as 1-octanol and 1-hexanol were obtained from Sigma Aldrich and acetone from QRec. Individual stock standard solutions of DBP and oleamide were each prepared at a concentration of 1mg/ml in methanol. Storage of stock solution of dibutyl phthalate was at 4$^{o}$C, whereas oleamide was at -20$^{o}$C. Working standards were freshly prepared everyday by diluting the stock solution with methanol.

## 2.3 Solvent Terminated Dispersive Liquid-Liquid Microextraction (ST-DLLME)

The ST-DLLME technique used to extract analytes of interest in this study was adapted from a method by Chen et al. (2010). 1g of honey was weighed, diluted to 5ml with distilled water, and vortexed to obtain a homogenous mixture. Aqueous honey was then filtered with a 0.45µm nylon filter into a glass test tube with conical bottom. A mixture of 50µL of extraction solvent, 1-hexanol and 500µL of disperser solvent, acetonitrile was rapidly injected into the

aqueous honey using a 1ml syringe. An emulsion was formed immediately. After a waiting time of 5 minutes, a second aliquot of 500μL disperser solvent acetonitrile functioning as a terminating solvent was added to the top layer of the honey sample to break the emulsion. Phase separation occurred on standing and the uppermost organic layer was withdrawn using a 1ml syringe and needle. This was then placed in the autosampler vial for HPLC analysis. Figure 2-1 below is a schematic diagram illustrating the ST-DLLME procedure.

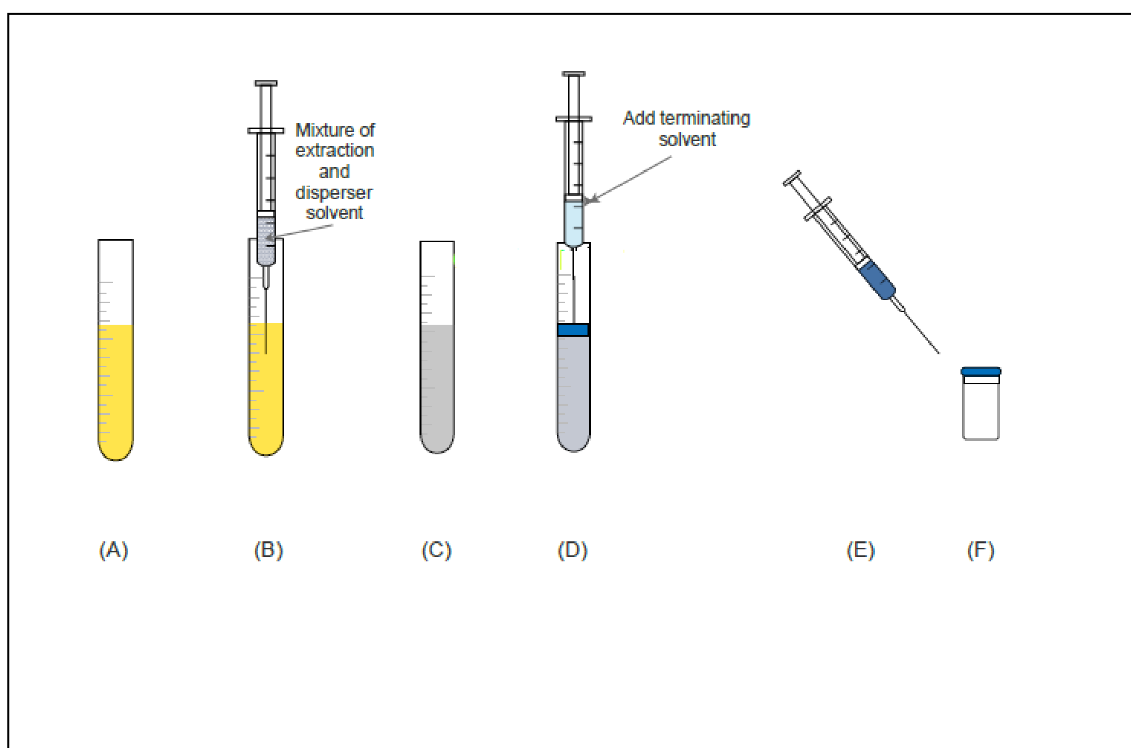


**Figure 2-1**: Steps in the ST-DLLME procedure.
(A) Aqueous honey containing the analytes of interest. (B) Rapid injection of a mixture of extraction and disperser solvent into aqueous honey. (C) Emulsion forms. (D) Addition of terminating solvent as a demulsifier; phase separation occurs. (E) Withdrawal of uppermost organic layer, (F) placed in autosampler vial for HPLC analysis.

## 2.4 Optimization of ST-DLLME

To achieve the optimized extraction condition for ST-DLLME, a few types of extraction solvent and disperser solvent were studied in combination, as shown in Table 2-1 below. Control honey was weighed, diluted with distilled water and filtered with a 0.45μm

nylon filter to remove particulate matter. It was then spiked with standards to produce a concentration of DBP at 5µg/g and oleamide at 10µg/g of honey. The volume of extraction solvent used was 50µL. The total volume of disperser solvent used was 1ml; its use separated into two parts. 0.5ml used initially together with extraction solvent to produce an emulsion, and the remainder 0.5ml added 5 minutes later to the emulsion to act as a terminating solvent or a demulsifier. All volumes of extraction and disperser solvent were kept constant throughout the experiment.

**Table 2-1**: The different combinations of extraction and disperser solvent used in the optimization of ST-DLLME.

| No. | Extraction Solvent | Disperser Solvent |
|---|---|---|
| 1 | 1-hexanol | Acetonitrile |
| 2 | | Acetone |
| 3 | | Methanol |
| 4 | 1-octanol | Acetonitrile |
| 5 | | Acetone |
| 6 | | Methanol |

To ensure that the DBP or oleamide detected were not of those introduced into the samples during the experiment; control honey without any spiked standards acting as a negative control also underwent extraction under exact similar experimental conditions. All glassware were thoroughly cleaned with acetone and dried in the oven overnight prior to use.

## 2.5 HPLC Analysis

High performance liquid chromatography was chosen as a method for simultaneous detection of both DBP and oleamide in the current study. HPLC was preferred to gas chromatography because one of the compounds of interest oleamide, does not volatilize easily and requires another derivatization step to aid its volatilization (Farajzadeh et al. 2006).

Chromatographic analysis was done using HPLC Varian Prostar 240 with Photodiode Array Detector equipped with an autosampler. A program Galaxie Chromatography Workstation on a personal computer was used to process chromatographic data. Stationary phase Zorbax Eclipse XDB-C18 column (4.6mm x 250mm, 5µm) from Agilent Technologies was used in the separation of analytes. The column temperature was maintained at 25°C throughout. Mobile phase used was acetonitrile: methanol at 70:30 v/v with isocratic elution. Flow rate was set at 1ml/min. The wavelength was set at 202nm. The autosampler withdraws 20µL of sample each time for analysis and the total run time was 10 minutes.

## 2.6 Optimization Parameters for HPLC Analysis

A mixed working solution containing both standards DBP (10µg/ml) and oleamide (300µg/ml) in methanol was prepared. Different types of mobile phase solvent combination and ratios were varied to obtain optimum separation and simultaneous identification of both analyte peaks. Mobile phase solvents that were used in the optimization include methanol, acetonitrile and deionized water.

Two different detector wavelengths at 202nm and 230 nm were also studied to identify the optimum wavelength of absorption by both analytes DBP and oleamide.

# Chapter 3 RESULTS

## 3.1 Optimization of HPLC Analysis

(a) Mobile Phase Solvents

The mobile phase solvent combinations acetonitrile, methanol and deionized water were varied in differing ratios to obtain good peak separation, the retention times of DBP and oleamide obtained are shown in Table 3-1.

**Table 3-1**: Retention time of analytes using differing ratios of mobile phase solvents.

| **Mobile Phase Ratios (v/v)** | | | **Retention time on chromatogram (min)** | |
|---|---|---|---|---|
| **Acetonitrile** | **Methanol** | **Deionized water** | **DBP** | **Oleamide** |
| - | 80 | 20 | 10.27 | ND |
| 5 | 65 | 30 | 7.21 | ND |
| 20 | 80 | - | 3.24 | ND |
| 80 | 20 | - | 2.95 | 3.47 |
| 70 | 30 | - | 3.39 | 6.07 |

Combination ratio of acetonitrile: methanol at 70:30 v/v gave the best peak separation within a considerable elution time of 10 minutes, with the chromatogram depicted in Figure 3-1. Peak identification with individual standards confirmed the retention time of DBP to be at 3.39 minutes, whereas oleamide was at 6.07 minutes, as shown in Figure 3-2 and Figure 3-3 respectively.

(b) Detector wavelength

Detector wavelength of 202nm and 230 nm were compared as part of the optimization parameter in this HPLC study. After comparing the HPLC chromatograms of both detector

wavelengths, 202nm was shown to have a better absorbance by both analyte DBP and oleamide. Hence, 202nm was chosen to be the detector wavelength for subsequent analysis.

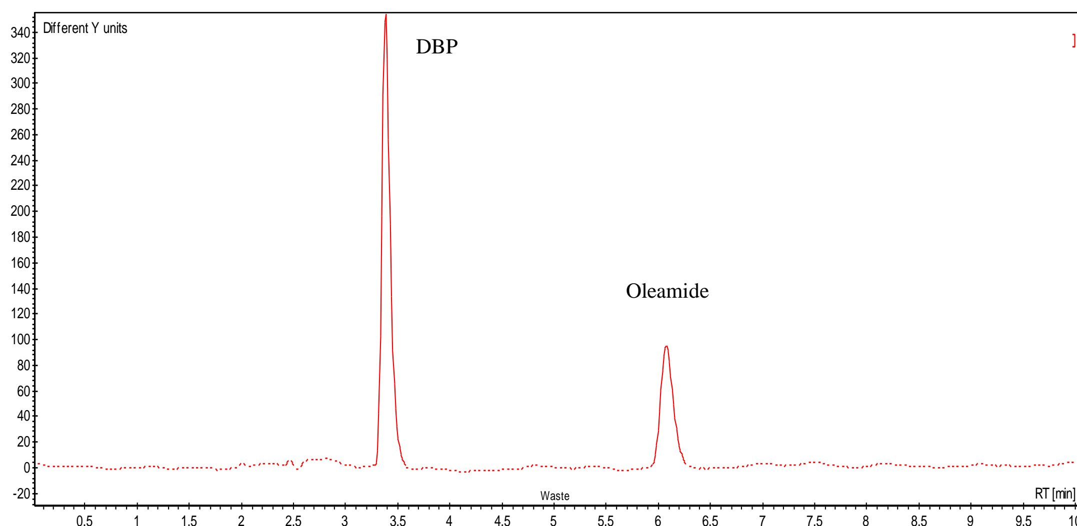


**Figure 3-1**: Chromatogram of separated analytes DBP and oleamide after optimization of mobile phase solvents and wavelength. Mobile phase acetonitrile: methanol 70: 30; wavelength 202nm.

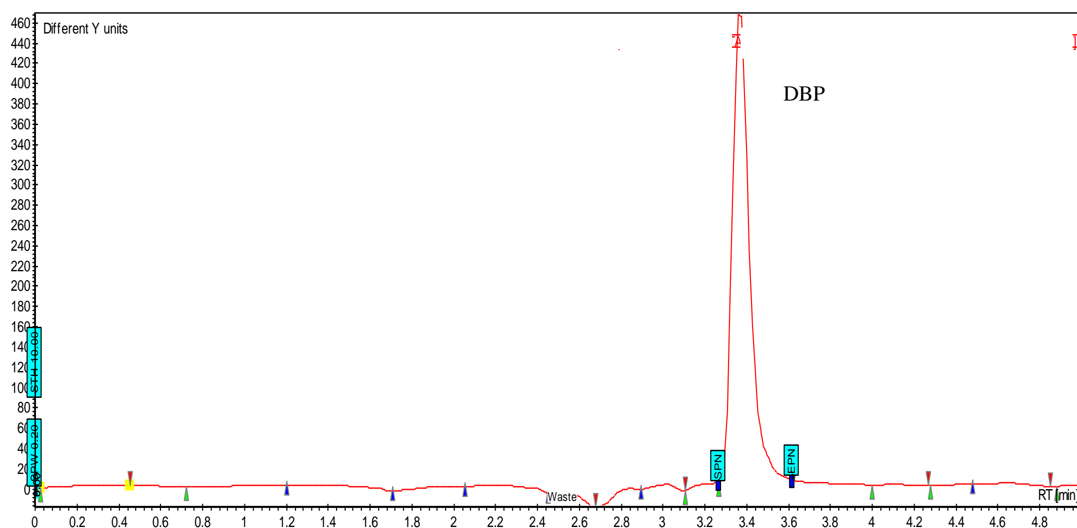


**Figure 3-2**: Confirmation of DBP peak at 3.39 minutes. Mobile phase acetonitrile: methanol 70: 30; wavelength 202nm.

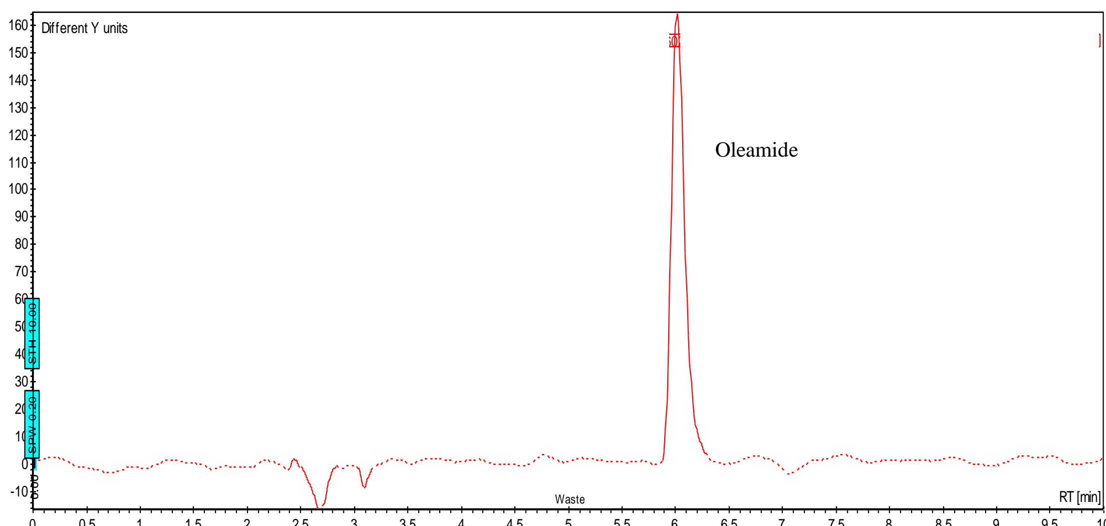

**Figure 3-3**: Confirmation of oleamide peak at 6.07 minutes, mobile phase 70: 30; wavelength 202nm.

## 3.2 Optimization of ST-DLLME

(a) Selection of Extraction and Disperser Solvent

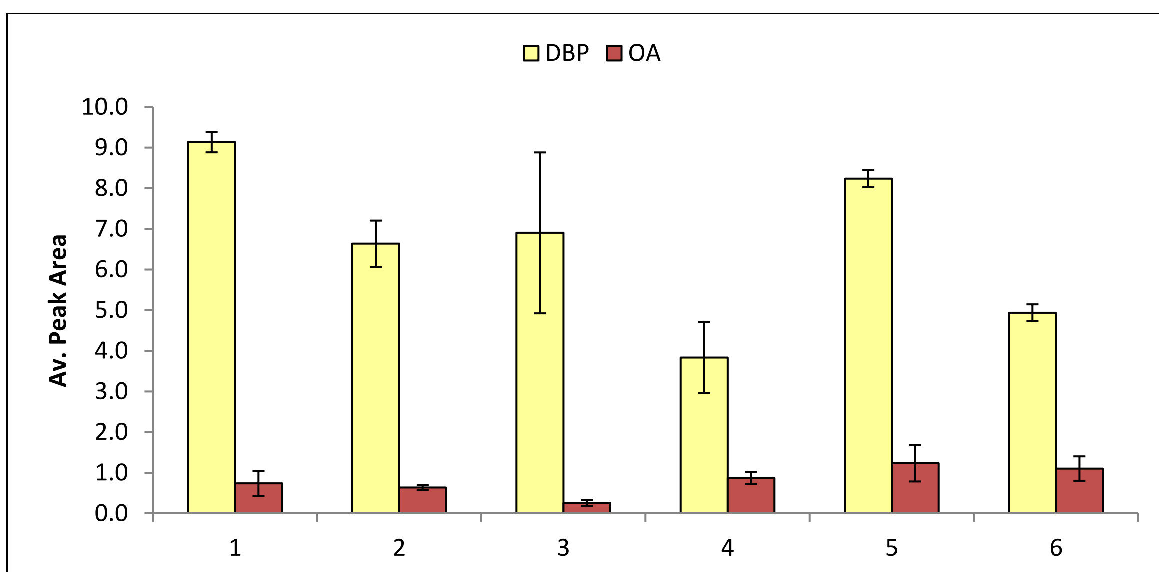

**Figure 3-4**: Average peak area absorption of DBP and oleamide using different extraction solvent and disperser solvent combinations.

Figure 3-4 demonstrates the effect of various combinations of extraction and disperser solvent on the simultaneous extraction of DBP and oleamide. Extraction solvent volume was fixed at 50µL, whereas the total volume of disperser solvent was fixed at 1ml. Spiked DBP and oleamide concentration in control honey was 5µg/g and 10µg/g respectively.

**Table 3-2**: Extraction results of DBP and oleamide with varying combinations of extraction and disperser solvent.

| No. | Extraction Solvent | Disperser Solvent | Average Peak Area | |
|---|---|---|---|---|
| | | | DBP | Oleamide |
| 1 | 1-hexanol | Acetonitrile | $9.13 \pm 0.25$ | $0.73 \pm 0.31$ |
| 2 | | Acetone | $6.63 \pm 0.56$ | $0.63 \pm 0.06$ |
| 3 | | Methanol | $6.90 \pm 1.97$ | $0.25 \pm 0.07$ |
| 4 | 1-octanol | Acetonitrile | $3.83 \pm 0.87$ | $0.87 \pm 0.15$ |
| 5 | | Acetone | $8.23 \pm 0.20$ | $1.23 \pm 0.45$ |
| 6 | | Methanol | $4.93 \pm 0.20$ | $1.10 \pm 0.30$ |

Table 3-2 is a tabulated result of the extraction efficiencies of different extraction and disperser solvent combinations on DBP and oleamide by ST-DLLME.

DBP was best extracted using mixture of 1-hexanol and acetonitrile (combination no.1) as it exhibited the largest peak area compared to others; while oleamide demonstrated greatest peak area when it was extracted with a mixture of 1-octanol and acetone (combination no.5).

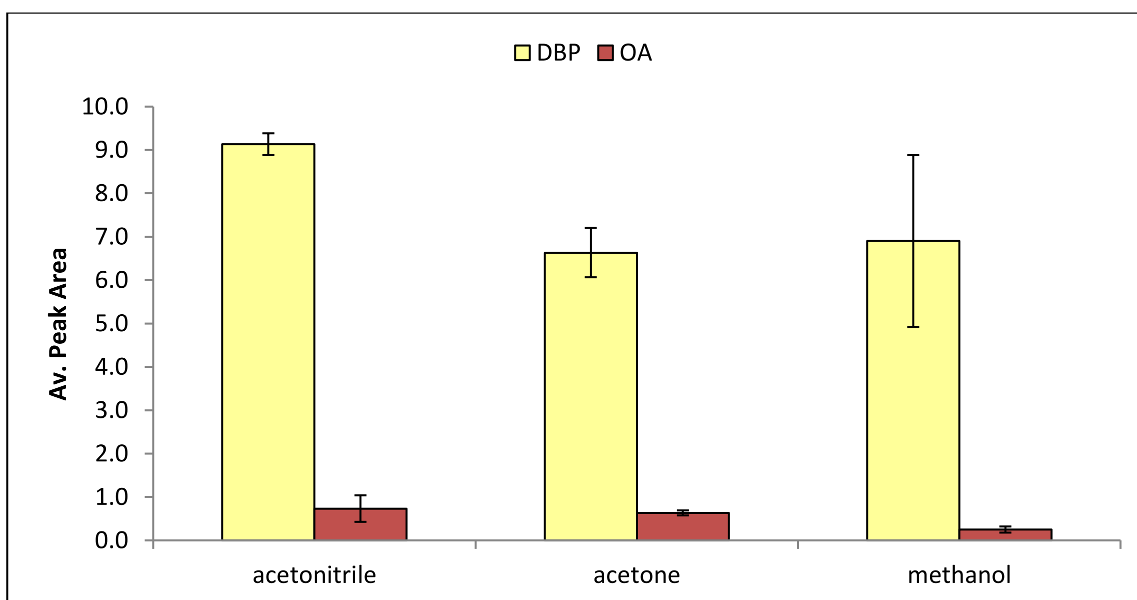


**Figure 3-5**: Comparison of extraction results between different disperser solvents; extraction solvent: 1-hexanol.

Figure 3-5 shows that when 1-hexanol was chosen as the extraction solvent, the use of acetonitrile as a disperser solvent produced a larger analyte extraction when compared to either acetone or methanol alone, as displayed by DBP and oleamide's higher peak area of absorption.

### 3.3 Evaluation of Method

(a) Standard Calibration Curves

Standard calibration curves were obtained by spiking a series of known analyte concentration into control honey, followed by extraction using optimized parameters of ST-DLLME. The graph was constructed by plotting average peak areas of analyte absorption against a series of known spiked concentrations.

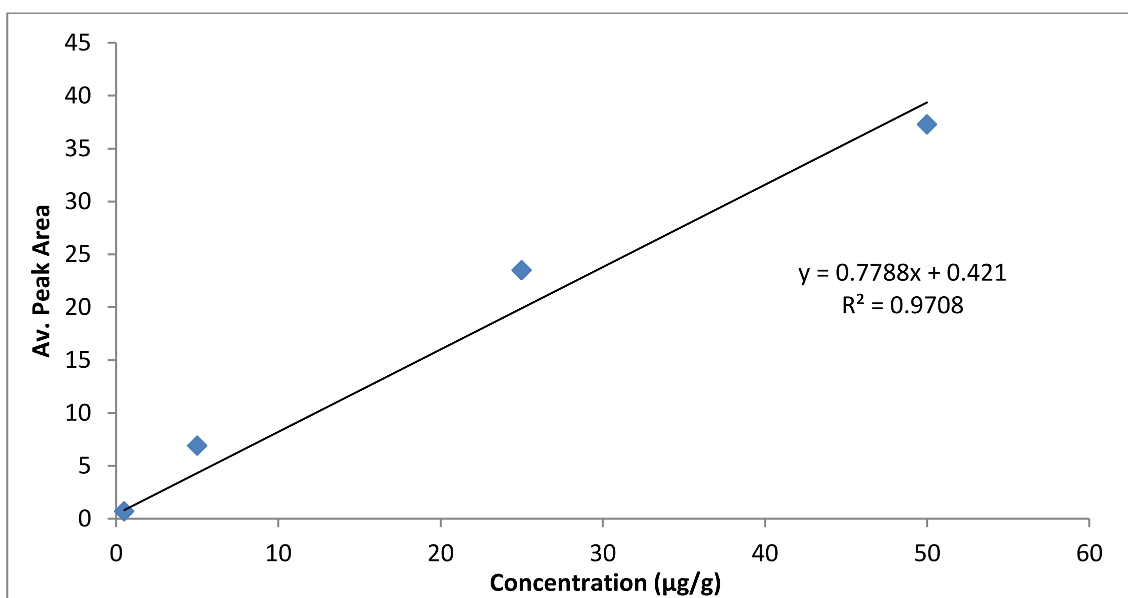


**Figure 3-6**: Standard calibration curve of DBP

The standard curve for DBP in Figure 3-6 has a linearity range between 0.5-50 µg/g. The coefficient of determination $R^2$ was 0.970.

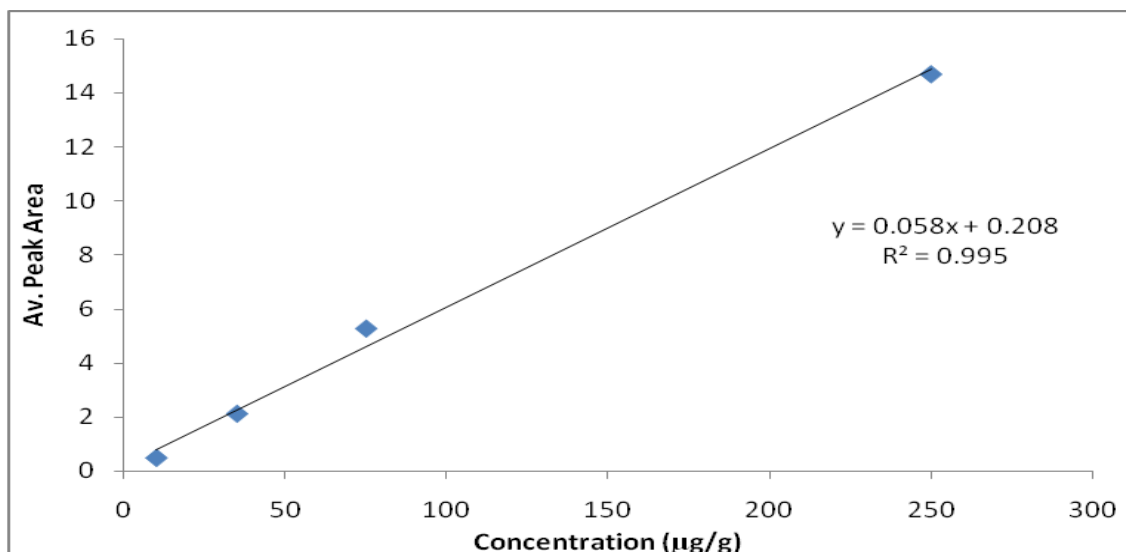


**Figure 3-7**: Standard calibration curve of oleamide.

Good linearity was obtained for the oleamide standard curve ranging from 10-250 µg/g with a coefficient of determination $R^2$ of 0.995, as exhibited in Figure 3-7.

(b) Limit of Detection and Limit of Quantitation

**Table 3-3**: Linear range, coefficient of determination ($R^2$), limit of detection (LOD) and limit of quantification (LOQ).

| **Analyte** | **Linearity Range (µg/g)** | **$R^2$** | **LOD (µg/g)** | **LOQ (µg/g)** |
|---|---|---|---|---|
| DBP | 0.5 – 50 | 0.970 | 0.15 | 0.5 |
| Oleamide | 10 – 250 | 0.995 | 4 | 11 |

The LOD and LOQ values for both DBP and oleamide are shown in Table 3-3. LOD and LOQ were estimated based on signal-to-noise ratio (S/N ratio), according to the International Conference on Harmonisation: Validation of Analytical Procedure (ICH 2005). LOD was estimated based on S/N ratio of 3; whereas LOQ was estimated based on S/N ratio of 10 (Shabir 2004; ICH 2005). The values approximated can be summarized into equations below:

$$\text{LOD} = \frac{\text{C}}{H/h} \times 3$$

$$\text{LOQ} = \frac{\text{C}}{H/h} \times 10$$

C: lowest achievable analyte concentration to produce a peak signal

H: Height of peak signal (at the lowest analyte concentration, C)

h: height of baseline noise (at the lowest analyte concentration, C)

## 3.4 Honey Sample Analysis

**Table 3-4**: Results of honey sample analysis.

| **Honey Samples** | **DBP** | **Oleamide** |
|---|---|---|
| B1 | ND | ND |
| B2 | ND | ND |
| B3 | ND | ND |
| P1 | ND | ND |
| P2 | ND | ND |
| P3 | ND | ND |
| P4 | ND | ND |
| P5 | ND | ND |
| P6 | ND | ND |

Control honey samples B1 to B3 were those drawn from hives with naturally made honey pots. Honey samples labelled as P1 to P6 were those taken from hives with artificial honey pots made of plastic. All honey samples were extracted using ST-DLLME and analyzed by HPLC as described in the method section earlier. Results in Table 3-4 showed that DBP and oleamide were not detected in all the honey samples.

# Chapter 4 DISCUSSION

HPLC is an instrument used for the separation and analysis of compounds that are non-volatile. Compound separation is possible due to each compound's unique affinity and interaction with the mobile phase and stationary phase of HPLC. In reverse phase HPLC, the stationary phase used is essentially hydrophobic; which are 18-carbon hydrocarbon chains attached to silica particles packed within the column. The mobile phase instead, is made up of solutions that are considerably more hydrophilic. Due to the concept of "like dissolves like", analytes that are hydrophobic will stay attached longer within the stationary phase column; which will elute later and produce a longer retention time. However, there are a few factors that affect the partitioning of analyte between the stationary and mobile phase. The intrinsic hydrophobicity of the analyte is one of them. This can be inferred from their individual log P values. Log P is the logarithmic value of the octanol-water partition coefficient. P represents the partition coefficient; which is described by the ratio of the concentration of a compound of interest in octanol to its concentration in water at equilibrium (Rutkowska et al. 2013). As both octanol and water are immiscible, the partitioning of a chemical compound between these two solvents at equilibrium is reflective of how hydrophobic a particular compound is. A higher log P value indicates greater hydrophobicity of the compound. In this study, the log P value of each analyte obtained can aid in predicting which analyte will be eluted first from the column.

Another factor is the composition of the mobile phase solvent. The extent of binding to the stationary phase and hence the elution time of analytes can be manipulated by altering the composition of mobile phases. Organic solvents such as acetonitrile and methanol can be used to reduce the mobile phase polarity and hasten the elution of hydrophobic analytes.

## 4.1 Optimization of HPLC Analysis

Mobile phase solvents were firstly optimized in this study to obtain a good separation of the two analyte peaks DBP and oleamide; and also to achieve a considerable elution time for both analytes. Peak assignment and identification was later carried out by referencing against chromatograms of individual pure standards DBP and oleamide obtained under identical analytical conditions as shown in Figure 3-2 and Figure 3-3.

In an attempt to separate the peaks of both analytes, the mobile phase initially used was methanol: deionized water at ratio 80:20 v/v to increase the polarity. However, only DBP was eluted at 10.27 minutes, and oleamide peak did not appear even though the run time has been increased to 15 minutes. When the combination was changed to a ratio of acetonitrile: methanol: deionized water at 5:65:30 with the aim to induce a slight reduction in polarity by introducing a small percentage of acetonitrile, not only was oleamide not detected on the chromatogram, the DBP peak at 7.21 minutes was also seen to be broad. Mobile phase was then changed to acetonitrile and methanol targeting at increasing the hydrophobicity of mobile phase to hasten analyte elution. Acetonitrile: methanol at ratio 20:80 v/v showed only the presence of DBP peak in the chromatogram at 3.24 minutes, with no sign of oleamide peak, a sign that probably oleamide was still attached to the stationary phase in the column. Increasing the ratio of acetonitrile to methanol at 80:20 v/v produced two analyte peaks that were too close together, which was at 2.95 minutes and 3.47 minutes. Peaks that are too close together may be difficult to identify when real samples are put through for analysis later. A summary of the mobile phases used and the analyte retention times are listed in Table 3-1.

A balance between short elution time and reasonable peak separation was achieved with acetonitrile: methanol at 70:30 v/v. Total run time was only 10 minutes with DBP appearing at 3.39 minutes and oleamide at 6.07 minutes. Oleamide eluted at a later time compared to DBP due to it being relatively more hydrophobic as predicted from its log P value. The log P value of

oleamide is 6.6 whereas for DBP, it is 4.72. A larger log P value signifies greater hydrophobicity, and hence stronger affinity for the stationary phase.

The chromatogram in Figure 3-1 shows the peak of DBP at a concentration of 10µg/ml and oleamide at 300µg/ml. The reason for choosing such a high oleamide concentration relative to DBP during peak separation was such that the oleamide peak appears comparable in size to its neighbouring peak DBP on the chromatogram. The possibility of oleamide not possessing as many chromophores as DBP may have contributed to its lack of UV absorption compared to DBP. Chemical structures of DBP and oleamide are shown in Figure 4-1 and Figure 4-2 below.

202nm and 230nm were detector wavelengths compared in this study. 230nm was the absorption wavelength used for HPLC analysis of a range of phthalate compounds including DBP (Shen et al. 2007). Stewart et al. 2002 and Farajzadeh et al. 2006 whom have analysed oleamide by HPLC at wavelength of 202nm, claimed that oleamide does not absorb beyond the range of 210nm. In this study, 202nm was selected as both DBP and oleamide appeared to absorb wavelength 202nm to a greater extent than 230nm.

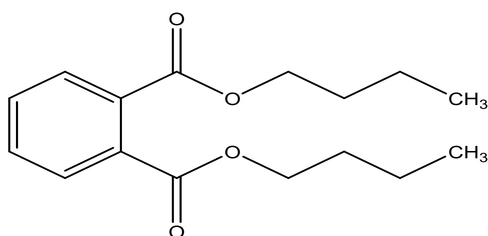


**Figure 4-1**: Chemical structure of DBP

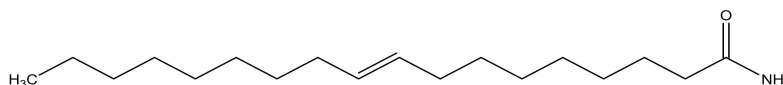


**Figure 4-2**: Chemical structure of oleamide