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Analysis of Dibutyl Phthalate and Oleamide in Stingless Bee Honey Harvested from Plastic Cups

(Analisis Dibutil Ftalat dan Oliamid pada Madu Lebah Kelulut yang Dituai daripada Cawan Plastik)

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ABSTRACT

Increasing demands for stingless bee honey in Malaysia have prompted alternative method using plastic cups as artificial honey pots within beehives to enhance yield. However, honey's acidity may lead to migration of plastic additives, affecting body's physiological responses on prolonged consumption. This study was performed to determine the presence of dibutyl phthalate (DBP) and oleamide in stingless bee honey collected from plastic cups. A 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. Good linearity was observed with coefficient of determination $R^2 \geq 0.97$ in the concentration range of 0.5-50 and 10-250 µg/g for DBP and oleamide, respectively. The limit of detection for DBP was 0.15 µg/g; while oleamide was 4 µg/g. The limit of quantitation for DBP and oleamide was 0.5 and 11 µg/g, respectively. DBP and oleamide were not detected in all the honey samples collected.

Keywords: Dibutyl phthalate; food safety; oleamide; stingless bee honey; ST-DLLME

ABSTRAK

Peningkatan permintaan bagi madu lebah kelulut di Malaysia telah menggalakkan kaedah alternatif menggunakan cawan plastik sebagai bekas madu tiruan dalam sarang lebah untuk meningkatkan hasil. Walau bagaimanapun, keasidan madu mungkin membawa kepada penghijrahan aditif plastik, mempengaruhi tindak balas fisiologi badan akibat penggunaan berpanjangan. Kajian ini dijalankan untuk menentukan kehadiran dibutil ftalat (DBP) dan oliamid dalam madu kelulut yang dikumpul daripada cawan plastik. Kaedah pengekstrakan serentak dan pengesanan oleh kedua-dua analit melalui pelarut tertamat pengekstrakan mikro cecair-cecair serakan (ST-DLLME) beserta dengan kromatografi cecair berprestasi tinggi (HPLC) telah dibangunkan. Kelinearan yang baik diperhatikan dengan pekali penentuan $R^2 \geq 0.97$ di dalam julat kepekatan 0.5-50 dan 10-250 µg/g masing-masing untuk DBP dan oliamid. Had pengesanan untuk DBP adalah 0.15 µg/g; manakala oliamid ialah 4 µg/g. Had pengkuantitian untuk DBP dan oliamid masing-masing adalah 0.5 dan 11 µg/g. DBP dan oliamid tidak dikesan dalam semua sampel madu yang dikumpul.

Kata kunci: Dibutil ftalat; keselamatan makanan; madu lebah kelulut; oliamid; ST-DLLME

INTRODUCTION

Honey is widely known for its naturally sweetening and medicinal properties (Pérez-pérez et al. 2013; Souza et al. 2006). Traditional and folklore medicine have long practised consuming honey for general wellbeing (Bogdanov et al. 2008; Kwakman & Zaat 2012). Local application of honey onto wound also aids recovery (Manyi-Loh et al. 2011). It does so through a few mechanisms; the presence of antioxidants such as the polyphenols provides anti-inflammatory benefits (Hasan et al. 2012) whereas hydrogen peroxide and the acidic pH contribute to its antibacterial effects (Bogdanov et al. 2008; Manyi-Loh et al. 2011). In addition, the acidity and high moisture content of honey aids in tissue regrowth (Manyi-Loh et al. 2011; Simon et al. 2009). A wide variety of honeys are produced by different tribes of bees. Stingless bees produce honey that are more sour and exotic in taste and can fetch a higher price on the market (Pimentel et al. 2013).

The increasing demands for stingless bee honey in Malaysia recently are due to the public's interest and awareness in consuming natural products for general wellbeing. Stingless bees depend very much on the forest for their natural habitat, as most colonies reside within the cavities of tree trunks. Increasing deforestation activities not only reduce the wild colonies of stingless bees (Oliveira et al. 2013), it also deprives the colonies of food (nectar and pollen) (Hiromitsu et al. 2004). Moreover, a colony of stingless bees only produces approximately 200 g to 5 kg of honey per year (Bradbear 2009).

In order to increase honey supply to meet the demands, beekeepers have brought beekeeping from the wild into their own farms. Wooden boxes are placed above the log hives that contain bees nest obtained from the forest to allow the colony to expand their honey and pollen pots within it. This ensures that honey can be harvested without damaging the nest, and also a sustained

production from the colony. Some beekeepers in Malaysia have taken the initiative of placing plastic cups lined in rows within the wooden boxes. These plastic cups mimic the shape and arrangement of the natural honey and pollen pots. The aim of providing ready-made honey storage pots was to encourage the stingless bees to increase their nectar foraging activity instead and hence increase honey production for commercial purposes.

Stingless bee honeys are known to have pH ranging between 3 and 4.5 (Souza et al. 2006). The plastic cups acting as artificial honey storage pots in commercial production are made of polypropylene material. Prolonged contact between the honey and plastic cup raises concerns that plastic additives may leach into the honey. The plastic additives of interest in this study are dibutyl phthalate (DBP) and oleamide, which are not covalently bonded to the polymer structure. The honey's acidity and high moisture content may affect their partitioning between the plastic cup and honey interface, hence contributing to the migration of harmful organic additives into the honey that people consume.

Phthalates are plasticizers used to impart strength and flexibility to plastics (Amayreh et al. 2013; Bhunia et al. 2013). They are found in toys, building materials and food packaging (Whyatt et al. 2014). Prenatal DBP exposure have shown an increased incidence of reproductive and developmental disorders in animal models, such as reduced anogenital distance, undescended testes in newborn male rats, cleft palate and skeletal malformations (U.S. EPA 2007). A significant association was also found between a reduction in anogenital distance in boys aged 2 to 36 months and their mother's prenatal urinary phthalate metabolite concentration in a multicentre cohort study across Los Angeles, Minneapolis and Columbia (Swan et al. 2005). Phthalates are also thought to contribute to endocrine diseases like diabetes and obesity; through the activation of peroxisome proliferator activated receptor pathway (Desvergne et al. 2009; James-Todd et al. 2012; Lind et al. 2012).

Another organic compound present in polypropylene plastics is oleamide, a fatty acid amide slip agent that facilitates plastic extrusion during manufacture (Bhunia et al. 2013). It is also an endogenous sleep inducing chemical in the cerebrospinal fluid of the brain, associated with the slow wave phase of the sleep cycle (Leggett et al. 2004; Solomonia et al. 2008). *In vivo* studies demonstrated that exogenous oleamide administration to mice and rats reduces body temperature and locomotion (Huitrón-Reséndiz et al. 2001), affects memory processes at doses 30-50 mg/kg (Murillo-Rodríguez et al. 2001) and significantly increases food intake (Martínez-González et al. 2004). There have been recent reports on the leaching of oleamide from disposable laboratory plastic ware made of polypropylene (Olivieri et al. 2012). This presents a food safety concern as the public may be exposed to these additives through honey consumption. Chronic exposure may affect our body's normal physiological processes.

The aim of this study was to measure DBP and oleamide in the commercially produced stingless bee honey. The honeys were harvested directly from the plastic cups placed within wooden box hives. The analytes were extracted using a solvent terminated dispersive liquid-liquid microextraction (ST-DLLME) method prior to high performance liquid chromatography (HPLC) analysis.

Materials and Methods

Honey samples

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

Reagents and standards

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

Sample preparation by 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). One g of honey was weighed, diluted to 5 mL with distilled water and vortexed to obtain a homogenous mixture. Aqueous honey was then filtered through a 0.45 µm nylon filter into a glass test tube with conical bottom. A mixture of 50 µL of extraction solvent, 1-hexanol and 0.5 mL 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 min, a second aliquot of 0.5 mL 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 where the uppermost organic layer was withdrawn using a 1 mL syringe and needle. This organic analyte was then injected into the

autosampler vial for HPLC analysis. Figure 1 is a schematic diagram illustrating the ST-DLLME procedure.

OPTIMIZATION OF ST-DLLME

In order to achieve the optimized extraction condition for ST-DLLME, a few extraction solvents and disperser solvents were studied in combination, as shown in Table 1. Control honey was weighed, diluted with distilled water and filtered through a 0.45 µm nylon filter to remove particulate matter. It was then spiked with standards to produce a concentration of DBP at 5 µg/g and oleamide at 10 µg/g of honey. The volume of extraction solvent used was 50 µL. The total volume of disperser solvent used was 1 mL; its use separated into two parts. 0.5 mL was used initially together with extraction solvent to produce an emulsion and the remainder 0.5 mL was added 5 min 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.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC was chosen as a method for simultaneous detection of both DBP and oleamide in honey after ST-DLLME. Chromatographic analysis was done using HPLC Varian Prostar 240 with Photodiode Array Detector equipped with an autosampler. Program software Galaxie Chromatography Workstation on a personal computer was used to process chromatographic data. The separation was performed on Zorbax Eclipse XDB-C18 column (4.6 mm × 250 mm, 5 µm) from Agilent Technologies. The column temperature was maintained at 25°C throughout. Mobile phase used was a mixture of acetonitrile: methanol at 70:30 v/v with isocratic elution. Flow rate was set at 1 mL/min. The detection was monitored at 202 nm. 20 µL of clean extract was withdrawn by the autosampler each time for analysis and the total run time was 10 min.

RESULTS AND DISCUSSION

ST-DLLME is an extraction method based on the formation of a ternary component system comprising of the extraction solvent, disperser solvent and also the aqueous sample (Amayreh et al. 2013). The rapid injection of the extraction and dispersive solvent mixture into the aqueous sample produces a rapid emulsification of oil droplets in water. Equilibrium can be achieved in a matter of seconds and presence of the many oil droplets constitutes a large surface area for extraction of the analyte of interest from the aqueous phase (Lv et al. 2013; Vasil et al. 2012). 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; and sufficiently concentrate the analytes to enable

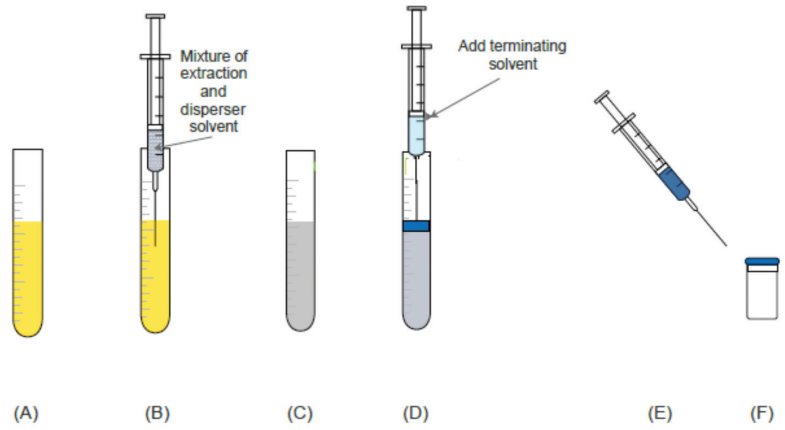

FIGURE 1. Steps in the ST-DLLME procedure (a) aqueous honey containing the analytes of interest, (b) rapid injection of a mixture of extraction and disperser solvent into aqueous honey, (c) emulsion forms, (d) addition of terminating solvent as a demulsifier; phase separation occurs, (e) withdrawal of uppermost organic layer and (f) placed in autosampler vial for HPLC analysis

TABLE 1. The different combinations of extraction solvent and disperser solvents used in ST-DLLME

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

detection by the instrument. Prior to the development of microextraction techniques, 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; Xueju et al. 2013).

OPTIMIZATION OF ST-DLLME PARAMETERS

Several parameters were optimized to produce a favourable condition for simultaneous extraction of DBP and oleamide. The type of extraction solvent and type of disperser solvent were optimized first before the analysis of honey samples. Spiked concentrations of DBP and oleamide in control honey were 5 and 10 µg/g, respectively. Optimum conditions were selected based on the average peak area of absorption produced by the analytes of interest in the HPLC chromatogram.

TYPE OF EXTRACTION SOLVENT

The extraction solvent used in ST-DLLME must have properties of low water solubility and is capable of extracting the analyte of concern (Vasil et al. 2012). Extraction solvents should also be either of higher or lower density than water such that when phase separation is induced, it can be easily withdrawn for further analysis. Early DLLME procedures use chlorinated solvents that have density higher than water as extraction solvents, but these solvents pose toxicity issues. Current alternatives use fatty alcohols that are more environmentally friendly but have a lower density than water (Çabuk et al. 2012). Therefore extraction solvents analysed in this method were 1-octanol and 1-hexanol.

Table 2 illustrates the comparison made between 1-hexanol and 1-octanol as extraction solvents. 1-hexanol demonstrated greater extraction efficiency for DBP when combined with disperser solvent acetonitrile (combination no. 1), which produced average peak area absorption of 9.13 $\pm$ 0.25. But oleamide was better extracted with 1-octanol in combination with disperser solvent acetone (combination no. 5); the average peak area of absorption being 1.23 $\pm$ 0.45.

Although the use of 1-octanol as extraction solvent generally produced a better oleamide extraction compared to 1-hexanol as evident from the results in Table 2, chromatograms produced by 1-octanol extraction showed broad peaks with peak tailing appearing behind the DBP peak. Hence, to achieve a balance between analyte enrichment and good peak separation in the chromatograms for both analytes, 1-hexanol was selected as the extraction solvent to be used for subsequent honey sample analysis.

TYPE OF DISPERSER AND TERMINATING SOLVENT

The disperser solvents used in DLLME should be miscible with both the extraction solvent and the aqueous phase (Vasil et al. 2012). Its role is in dispersing the extraction solvent as tiny oil droplets in the emulsification stage thus creating a large surface area to enable the rapid transfer of analytes into the extraction solvent (Çabuk et al. 2012). The first aliquot of disperser solvent was added as a mixture with the extraction solvent into the aqueous sample functions to create an emulsion. The addition of a second aliquot acts as a terminating solvent to destabilize the emulsion resulting in phase separation (Chen et al. 2010). This solvent terminating step omits the need for centrifugation as a mean of separating the organic phase from the aqueous phase (Guo & Lee 2011). Acetonitrile, acetone and methanol are solvents with these properties and were therefore investigated as part of the optimization process in this study.

With 1-hexanol 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 in Table 2. The average peak area absorption of DBP using disperser solvent acetonitrile was 9.13 $\pm$ 0.25; followed by methanol 6.9 $\pm$ 1.98; and then acetone 6.63 $\pm$ 0.57. Average peak area absorption for oleamide when the disperser solvent was acetonitrile was 0.73 $\pm$ 0.31; followed by acetone 0.63 $\pm$ 0.06; and then methanol at 0.25 $\pm$ 0.07. Thus, acetonitrile was chosen as the disperser solvent and used in subsequent experiments.

OPTIMIZATION OF HPLC ANALYSIS

The mobile phase solvent combinations acetonitrile and methanol were varied in differing ratios to obtain good peak separation. Combination ratio of acetonitrile: methanol at 70:30 v/v gave the best peak separation within a considerable elution time of 10 min. Peak identification with individual standards confirmed the retention time of DBP to be at 3.39 min, whereas oleamide was at 6.07 min as shown in Figure 2.

Detector wavelength of 202 and 230 nm were compared as part of the optimization parameter in this HPLC study. 230 nm was the absorption wavelength used by Shen et al. (2007) in the HPLC analysis of a range of phthalate compounds including DBP. Farajzadeh et al. (2006) and Stewart et al. (2002) who have analysed oleamide by HPLC at wavelength of 202 nm, showed that oleamide does not absorb beyond the range of 210 nm. After comparing the HPLC chromatograms produced at both detector wavelengths, 202 nm was shown to have a better absorbance by both analyte DBP and oleamide. Hence, 202 nm was selected as the detector wavelength for subsequent analysis.

EVALUATION OF METHOD

Matrix-matched Calibration Curves The matrix-matched calibration curves were obtained by spiking a series of

TABLE 2. Extraction results of various combinations of extraction and disperser solvents

No.	Extraction solvent	Disperser solvent	Average peak area	
			DBP	Oleamide
1	1-hexanol	Acetonitrile	9.13 ± 0.25	0.73 ± 0.31
2		Acetone	6.63 ± 0.56	0.63 ± 0.06
3		Methanol	6.90 ± 1.97	0.25 ± 0.07
4	1-octanol	Acetonitrile	3.83 ± 0.87	0.87 ± 0.15
5		Acetone	8.23 ± 0.20	1.23 ± 0.45
6		Methanol	4.93 ± 0.20	1.10 ± 0.30

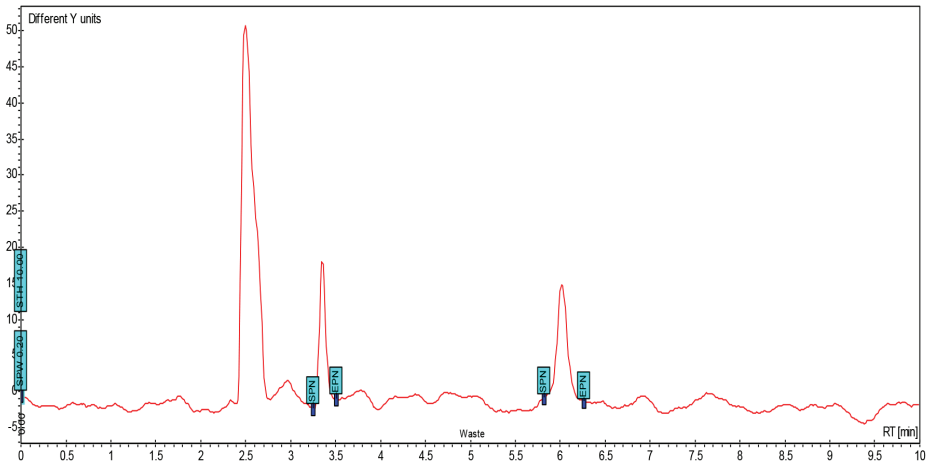

FIGURE 2. Chromatogram of spiked standards DBP and oleamide

known amounts of DBP and oleamide into the control honey followed by extraction using ST-DLLME. It is a graphical plot of average peak area of the extracted analyte against a series of known spiked analyte concentrations.

DBP working concentrations were prepared in honey in the range of 0.5 - 250 µg/g. Linearity was obtained from 0.5 - 50 µg/g with a coefficient of determination R^2 of 0.970. Oleamide was spiked into honey to obtain concentrations ranging between 10 and 250 µg/g, and linearity was also achieved within the same range with R^2 value of 0.995.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD of an analyte is the lowest concentration that can be detected by an analytical method (United Nations Office on Drug and Crime 2009). It is a concentration that only denotes the presence of the analyte of interest but is unable to quantify it. LOQ is the lowest concentration of an analyte that can be quantified within an analytical procedure with acceptable accuracy and precision (Shrivastava & Gupta 2011). 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). The LOD and LOQ values for both DBP and oleamide are shown in Table 3.

HONEY SAMPLE ANALYSIS

Some plastic additives have threshold limits which have been pre-determined by authorities concerned pertaining to the amounts that can migrate from food contact materials. These are the levels which permit the presence of small amounts of additives in food, but are still considered safe for human consumption. According to the EU Commission No. 10/2011, the safety limit for DBP as defined by its specific migration limit (SML) in food is 0.3 mg/kg. Specific migration limits are applicable to food contact materials containing food additives or polymer production aids that have the potential of leaching into food. It is the maximum amount of substance permissible per kg of food. However, oleamide has no SML value specified in this regulation. Under circumstances if an additive is permitted to be used as a polymer production aid and where there are no restrictions provided, a general value of 60 mg/kg shall be applied. In the method evaluation step, the LOD for DBP was 0.15 and oleamide 4 mg/kg, respectively, which were both lower than the SML provided by the EU Commission. This demonstrated that the method developed was sensitive enough to detect the threshold limits of DBP and oleamide in the collected honey samples.

Control honeys B1 to B3 drawn from hives with naturally made honey pots act as negative controls. Honey samples taken from hives with artificial honey pots made of plastic were labelled as P1 to P6. All honey samples were extracted using ST-DLLME and analyzed by HPLC under the optimized conditions. The result in Table 4 shows that DBP and oleamide were not detected in all the honey samples.

TABLE 3. Linearity range, coefficient of determination, R^2, LOD and 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

TABLE 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

ND: Not Detected

CONCLUSION

This study did not detect the organic contaminants namely DBP and oleamide in stingless bee honey cultivated from plastic cups. An extraction method by ST-DLLME was developed and optimized for the extraction and pre-concentration of both DBP and oleamide from stingless bee honey. This step was followed by separation and detection of analytes by HPLC. The linearity range, with the LOD and LOQ values applicable for both analytes were established in this study. ST-DLLME coupled with HPLC presents a sensitive and reliable method for the detection of DBP and oleamide in stingless bee honey, with the ability to detect concentrations below the specified threshold limits.

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Yee Ping Koo collected test data and drafted the manuscript with Noorfatimah Yahaya. Wan Adnan Wan Omar designed the study and interpreted the results. The authors declare that there are no conflicts of interest.

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