GRAPHENE-MAGNETITE AS MAGNETIC SOLID PHASE ADSORBENT FOR EXTRACTION OF 4-HYDROXYBENZOIC ACID AND 3,4-DIHYDROXYBENZOIC ACID IN STINGLESS BEE HONEY

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ABSTRACT

Stingless bee honeys are rich in secondary metabolites such as free phenolic acids which can be easily absorbed into the body. Trace amount of the phenolic acids had made the analysis difficult, hence sample pretreatment is crucial. In this work, a graphene-magnetite composite (G-Fe_3O_4) was synthesized and assessed as an adsorbent for magnetic solid phase extraction (MSPE) of two phenolic acids namely 4-hydroxybenzoic acid (4-HB) and 3,4-dihydroxybenzoic acid (3,4-DHB) from honey samples prior to analysis using high performance liquid chromatography with ultraviolet-visible detector (HPLC-UV/Vis). Characterizations of G-Fe_3O_4 were performed using Fourier transform infrared spectroscopy (FTIR), low vacuum scanning electron microscopy (LVSEM) and nitrogen adsorption analysis. Several MSPE parameters affecting the extraction of these two phenolic acids were optimized. Optimum MSPE conditions were 50 mg of G-Fe_3O_4 adsorbent, vortex rotational speed of 1600 rpm, 5 min extraction time, 30 mL sample volume at pH 0.5, 200 µL methanol as desorption solvent (5 min sonication assisted) and 5% w/v NaCl salt. Matrix-matched calibration was used for the analysis of the two phenolic acids from several honey samples. Calibration graphs were linear in the range 1–50 µg/g (R^2 = 0.9997) for 4-HB and 3–50 µg/g (R^2 = 0.9996) for 3,4-DHB. The limit of detection (LOD = 3S/N) calculated for 4-HB and 3,4-DHB was 0.08 µg/g and 0.14 µg/g, respectively. Good relative recoveries (72.6-110.6%) were obtained for both phenolic acids from honey samples with RSD < 6.0% (*n* = 3). The developed G-Fe_3O_4 MSPE method was applied to the analysis of both phenolic acids in honey samples from Johor Bahru, Johor. Two *Trigona* spp. honey samples (H1 and H2) and a commercial honey sample (H3) were used in this study. The amount of 4-HB and 3,4-DHB in H1 sample were 0.14 ± 0.9 µg/g and 0.67 ± 1.7 µg/g honey, respectively. H2 sample showed slightly higher amount of both phenolic acids (0.47 ± 3.1 µg/g for 4-HB and 1.61 ± 2.3 µg/g for 3,4-DHB). The amount of 4-HB and 3,4-DHB extracted from H3 sample was below the LOD of the developed method. The developed G-Fe_3O_4 MSPE method offered is simple, environmental friendly and efficient for extraction of phenolic acids from honey samples.

ABSTRAK

Madu lebah kelulut kaya dengan metabolit sekunder seperti asid fenolik bebas yang mudah diserap oleh tubuh. Kuantiti surih asid fenolik menyukarkan proses analisis, oleh itu pra-rawatan sampel adalah penting. Dalam kajian ini, komposit grafin-magnetit (G-Fe_3O_4) telah disintesis dan dinilai sebagai penjerap untuk pengekstrakan fasa pepejal magnet (MSPE) dua asid fenolik iaitu asid 4-hidroksibenzoik (4-HB) dan asid 3,4-dihidrosibenzoik (3,4-DHB) daripada sampel madu sebelum analisis menggunakan kromatografi cecair berprestasi tinggi dengan pengesan ultralembayung-nampak (HPLC-UV/Vis). Pencirian G-Fe_3O_4 telah dibuat menggunakan spektroskopi inframerah transformasi Fourier (FTIR), mikroskopi imbasan electron vakum rendah (LVSEM) dan analisis penjerapan nitrogen. Beberapa parameter MSPE yang mempengaruhi pengekstrakan kedua-dua asid fenolik ini telah dioptimumkan. Keadaan optimum MSPE ialah 50 mg penjerap G-Fe_3O_4, 1600 rpm kelajuan putaran vortek, 5 min masa pengekstrakan, 30 mL isipadu sampel pada pH 0.5, 200 µL metanol sebagai pelarut penyaherapan (5 min bantuan sonikasi) dan 5% w/v garam NaCl. Graf kalibrasi matrik berpadan telah digunakan untuk analisis kedua-dua asid fenolik tersebut daripada beberapa sampel madu. Graf kalibrasi adalah linear dalam julat 1-50 µg/g (R^2 = 0.9997) untuk 4-HB dan 3-50 µg/g (R^2 = 0.9996) untuk 3,4-DHB. Had pengesanan (LOD = 3S/N) yang dihitung untuk 4-HB dan 3,4-DHB masing-masing ialah 0.08 µg/g dan 0.14 µg/g. Perolehan semula relatif yang baik (72.6-110.6%) diperoleh untuk kedua-dua asid fenolik daripada sampel madu dengan RSD < 6.0% (*n* = 3). Kaedah G-Fe_3O_4 MSPE yang dibangunkan telah diaplikasikan kepada analisis kedua-dua asid fenolik dalam beberapa sampel madu dari Johor Bahru, Johor. Dua sampel madu daripada *Trigona* spp. (H1 dan H2) dan sampel madu komersial (H3) telah digunakan dalam kajian ini. Kandungan 4-HB dan 3,4-DHB dalam sampel H1 masing-masing ialah 0.14 ± 0.9 µg/g dan 0.67 ± 1.7 µg/g madu. Sampel H2 menunjukkan kandungan yang lebih tinggi untuk kedua-dua asid fenolik (0.47 ± 3.1 µg/g for 4-HB dan 1.61 ± 2.3 µg/g untuk 3,4-DHB). Amaun 4-HB dan 3,4-DHB yang diekstrak daripada sampel H3 adalah di bawah LOD kaedah yang dibangunkan. Kaedah G-Fe_3O_4 MSPE yang dibangunkan adalah ringkas, mesra alam dan berkesan untuk pengekstrakan asid fenolik daripada sampel madu.

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LIST OF ABBREVIATIONS

µ-SPE	-	Micro-solid phase extraction
3,4-DHB	-	3,4-Dihydroxybenzoic acid
3D-G@Fe_3O_4	-	Three-dimensional graphene nano-composite
4-HB	-	4-Hydroxybenzoic acid
C.	-	Concentration
CCG	-	Chemically-converted graphene
CE	-	Capillary electropherosis
CE-DAD	-	Capillary electrophoresis-diode array detector
CE-PDA	-	Capillary electrophoresis-photodiode array detector
CH_3CN	-	Acetonitrile
c-MWCNT-MNPs	-	Magnetic carboxylated multi-walled carbon nanotubes
CO	-	Carbon monoxide
CO_2	-	Carbon dioxide
CTAB	-	Cetyltrimethylammonium bromide
CVD	-	Chemical vapour deposition
DMSPE	-	Dispersive micro solid-phase extraction
Fe	-	Iron
Fe_3O_4	-	Magnetite/iron (II, III) oxide
Fe_3O_4/SiO_2	-	Magnetite silica
Fe_3O_4@(P-*co*-EDMA)	-	Magnetic poly(diethyl vinylphosphonate-co-ethylene glycol dimethacrylate)
Fe_3O_4@C	-	Carbon-coated magnetite
Fe_3O_4@C@PANI	-	Carbon-coated magnetite polyaniline
Fe_3O_4@G-TEOS-MTMOS	-	Graphene-based tetraethoxysilane-methyltrimethoxysilane sol-gel hybrid magnetic

		nanocomposite
Fe_3O_4@Mg-Al LDH	-	Magnesium–aluminum layered double hydroxide coated on magnetic nanoparticles
$FeCl_2.4H_2O$	-	Iron (II) chloride tetrahydrate
$FeCl_3$	-	Iron trichloride
$FeCl_3.6H_2O$	-	Iron (III) chloride hexahydrate
FTIR	-	Fourier transform infrared spectroscopy
GC-µECD	-	Gas chromatography-micro electron capture detector
g-C_3N_4/Fe_3O_4	-	Graphitic carbon nitride nanocomposite with magnetite
GC-ECD	-	Gas chromatography-electron capture detector
GC-MS	-	Gas chromatography-mass spectrometry
GC-NPD	-	Gas chromatography-nitrogen phosphorus detector
G-Fe_3O_4	-	Graphene-magnetite
GO	-	Graphene oxide
h	-	Hours
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
HMF	-	Hydroxymethylfurfural
HPLC	-	High-performance liquid chromatography
HPLC-DAD	-	High-performance liquid chromatography-diode array detector
HPLC-FLD	-	High-performance liquid chromatography-fluorescence detector
HPLC-PDA	-	High-performance liquid chromatography-photodiode array detector
HPLC-UV	-	High-performance liquid chromatography-ultraviolet detector
HPLC-UV/Vis	-	High-performance liquid chromatography-ultraviolet-visible detector
HPLC-VWD	-	High-performance liquid chromatography-variable wavelength detector
HPMIPs	-	Hollow porous molecularly imprinted polymers

IPA	-	Isopropanol
KBr	-	Potassium bromide
KH_2PO_4	-	Potassium dihydrogen phosphate
$KMnO_4$	-	Potassium permanganate
LC-MS/MS	-	Liquid chromatography/tandem mass spectrometry
Li_2O	-	Lithium oxide
LLE	-	Liquid-liquid extraction
LOD	-	Limit of detection
LOQ	-	Limit of quantification
LVSEM	-	Low vacuum scanning electron microscopy
MARDI	-	Malaysian Agricultural Research and Development Institute
MEKC-UV	-	Micellar electrokinetic chromatography-ultraviolet detector
MeOH	-	Methanol
min	-	Minute
MNPs	-	Magnetic nanoparticles
MSPE	-	Magnetic solid phase extraction
MWCNTs/Fe_3O_4@PPy	-	Multiwalled carbon nanotubes magnetite-polypyrrole
MWNTs@Fe_3O_4-MIPs	-	Magnetic multi-walled carbon nanotubes molecularly imprinted polymer
N_2	-	Nitrogen
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
NH_4OH	-	Ammonium hydroxide
R	-	Recovery
rpm	-	Rate per minute
RR	-	Relative recovery
RSD	-	Relative standard deviation
RTILs	-	Room temperature ionic liquids
RTILs-coated Fe_3O_4/SiO_2	-	Room temperature ionic liquids coated magnetite silica

SAME	-	Solvent-assisted microwave extraction
SEM	-	Scanning electron microscopy
SFE	-	Supercritical fluid extraction
Si	-	Silicon
SiC	-	Silicon carbide
SiO2	-	Silica
SPE	-	Solid-phase extraction
SPME	-	Solid-phase microextraction
T.	-	*Trigona*
UE	-	Ultrasonic extraction
UHPLC-MS/MS	-	Ultra high performance liquid chromatography-tandem mass spectrometry
UHPLC-Q-TOF/MS	-	Ultra high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

LIST OF SYMBOLS

Log $K_{O/W}$	-	Log octanol/water partition coefficient
t_R	-	Retention time
pKa	-	Acid ionization constant
R^2	-	Coefficient of determination
π	-	Pi

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Stingless bees originated from the tribe of Meliponini and widely distributed in the tropical regions. About 32 species have been documented in Malaysia with the *Trigona* being the largest genus found (Norowi *et al.,* 2010). Study on stingless bee honey has emerged due to its unique properties such as having unusual degree of acidity, sweetness, sourness and most importantly is high medicinal value (Vit *et al.,* 2013). The composition of stingless bee honey includes the mixture of carbohydrates, with fructose and glucose as the major constituents, water and phytochemicals (Jaapar *et al*., 2016). The presence of phytochemicals including phenolic acids, flavonoids, vitamins, minerals, lipids and enzymes has contributed to its therapeutic effects (Silva *et al.,* 2013).

Phenolic acids are the secondary metabolites widely distributed in plants which exert potent antioxidant properties higher than those in vitamin C and E (Tsao and Deng, 2004). Moreover, they also act as protective agents against diseases associated with oxidative damage (Robbins, 2003). They can exist as free, esterified or insoluble-bound form (Stalikas, 2007). Research on the composition of free phenolic acids in stingless bee honey has been a great field to study due to their ability to be easily absorbed by the body hence perform various pharmacological activities (Roowi *et al.,* 2012).

Several methods have been demonstrated for the extraction of phenolic acids from various matrices. These include liquid-liquid extraction (LLE) (Pancorbo *et al.*, 2004; Plessi *et al.*, 2006; Zgórka and Kawka, 2001), supercritical fluid extraction (SFE) (Chang *et al.*, 2001), ultrasonic extraction (UE), solvent-assisted microwave extraction (SAME) (Pomponio *et al.*, 2002) and solid-phase extraction (SPE) (Pyrzynska and Biesaga, 2009). SPE is the most common method used in various applications. However, some drawbacks including high sample volume and time-consuming have limited its use (Buszewski and Szultka, 2012). Therefore, modification of the SPE technique through the introduction of magnetic nanoparticles (MNPs) as adsorbent has been established to propose a more simple and efficient method known as magnetic solid phase extraction (MSPE) (Šafaříková and Šafařík, 1999).

However, magnetic nanoparticles promote some limitations due to its aggregation, low stability in acidic medium and easily oxidized which leads to their modifications and developments. These provide a more efficient method at the same time enhance the selectivity of the modified adsorbent towards the target analytes (Wan Ibrahim *et al.*, 2015). Over the years, several developments on the MSPE adsorbents have been reported (Abd Ali *et al.*, 2016; Ibarra *et al.*, 2015; Kamboh *et al.* 2016; Nodeh *et al.*, 2016; Nodeh *et al.*, 2017; Wan Ibrahim *et al.*, 2015) whereas using graphene as adsorbent had become a great interest (Liu *et al.*, 2012). Graphene has high surface area of theoretically 2630 m^2/g (Stoller *et al.*, 2008) and comprised of large delocalized π-electron structure. Therefore, the combination of graphene and magnetite (Fe_3O_4) to produce graphene-magnetite (G-Fe_3O_4) adsorbent is expected to promote high surface area for adsorption and ease of separation (Wang *et al.*, 2012).

To date, little evidence has been found regarding the use of graphene-magnetite (G-Fe_3O_4) as adsorbent for the extraction of phenolic acids. Theoretically, G-Fe_3O_4 has great potential as adsorbent for phenolic acids through the hydrophobic and π-interactions. In this study, the suitability of G-Fe_3O_4 as an adsorbent for MSPE of selected free phenolic acids in *Trigona* spp. honey was assessed prior to analysis using high-performance liquid chromatography coupled with ultraviolet-visible detector (HPLC-UV/Vis).

1.2 Problem Statement

Stingless bee honey is well-known as a highly nutritional food, with the presence of phenolic acids as part of the pharmacologically active compounds (Jaapar *et al.*, 2016). However, the amounts are not widely known due to improper labelling and lack of scientific research. Although there is no specific legislation of these compounds in food, Malaysian labelling regulation requires that nutrient and health claims should be based on scientific findings (Expert Committee on Nutrition, Health Claims and Advertisement, 2010). Small amount of phenolic acids have made their analysis become a great challenge. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) had been widely used. However, both methods promote several limitations. The conventional LLE is tedious and required high consumption of toxic organic solvent. SPE had been applied in various samples but the method is time consuming, expensive and might promote channeling effect. Other methods include ultrasonication extraction (UE) and dispersive micro-solid phase extraction (DMSPE), a developed SPE method. UE might lead to degradation of the targeted compounds due to long irradiation time. DMSPE promote a selective method with low consumption of organic solvent. However, centrifugation and filtration will be required which makes the process tedious. Therefore, finding the most suitable pre-concentration method for phenolic acids have emerged with several factors to be taken into considerations including their efficiency, selectivity, sensitivity and most importantly environmental friendly. As such, MSPE of the selected phenolic acids using G-Fe_3O_4 as an adsorbent is expected to fulfil the requirements.

1.3 Aims and Objectives of Study

The aims of this study are to synthesize and apply G-Fe_3O_4 as an adsorbent to extract two selected phenolic acids in *Trigona* spp. honey prior to analysis using HPLC-UV/Vis. The objectives of this study are to:

i. prepare G-Fe_3O_4 nanoparticles followed by characterizations using Fourier transform infrared spectroscopy (FTIR), low vacuum scanning electron microscopy (LVSEM) and nitrogen (N_2) adsorption analysis.
ii. optimize the MSPE parameters for 4-hydroxybenzoic acid (4-HB) and 3,4-dihydroxybenzoic acid (3,4-DHB).
iii. validate the developed G-Fe_3O_4 MSPE method.
iv. apply the G-Fe_3O_4 as an adsorbent for MSPE of 4-HB and 3,4-DHB prior to quantification using HPLC-UV/Vis by applying the developed G-Fe_3O_4 method to the analysis of honeys from stingless and honey bee for comparison.

1.4 Scope of Study

Two stingless bee honey samples were used in this study, both originated from the *Trigona* spp. obtained from the beekeepers in Johor, Malaysia. G-Fe_3O_4 adsorbent was prepared and characterized using FTIR, LVSEM and N_2 adsorption analysis. Optimization of the MSPE conditions using G-Fe_3O_4 adsorbent towards the two selected phenolic acids namely 4-hydroxybenzoic acid (4-HB) and 3,4-dihydroxybenzoic acid (3,4-DHB) was performed for sample pH, type of desorption solvent, mass of adsorbent, extraction time, desorption time, vortex rotational speed, sample volume, volume of desorption solvent and salt addition (NaCl). The adsorption performance of G-Fe_3O_4 towards the extraction of selected phenolic acids was investigated through comparison with graphene and Fe_3O_4. Moreover, the reusability of the prepared G-Fe_3O_4 was studied until there was no significant change in the peak area. The G-Fe_3O_4 adsorbent was then applied for the MSPE of 4-HB and 3,4-DHB in *Trigona* spp. honey samples under optimum conditions followed by analysis using HPLC-UV/Vis. Similar step was performed towards a commercial honey sample from honey bee for comparison.

1.5 Significance of Study

Free phenolic acids are easily absorbed into the body hence perform various pharmacological activities. However, their analysis in honey samples has become challenging due to the traces amount which made the pre-concentration step become crucial. Since the claims of health and nutrition in food products must be based on recent scientific findings, this research would be a good contribution. In this study, pre-concentration of the selected free phenolic acids was performed using G-Fe_3O_4 as adsorbent for MSPE followed by quantification using HPLC-UV/Vis. Hence, useful information on the constituents of the free phenolic acids in stingless bee honey can be provided to the consumer through proper labelling of the products as stated in Malaysian Standard, MS1529:2015 (Plant-based organically produced foods-Requirements for production, processing, labelling and marketing) (Draft Malaysian Standard, 2014). In addition, the prepared G-Fe_3O_4 MSPE method provides a faster and easier approach for analysis of free phenolic acids compared to LLE and SPE.

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