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Graphene-magnetite as adsorbent for magnetic solid phase extraction of 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid in stingless bee honey

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ABSTRACT

Graphene-magnetite composite (G-Fe_3O_4) was successfully synthesized and applied as 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 stingless bee honey prior to analysis using high performance liquid chromatography with ultraviolet–visible detection (HPLC-UV/Vis). Several MSPE parameters affecting extraction of these two acids were optimized. Optimum MSPE conditions were 50 mg of G-Fe_3O_4 adsorbent, 5 min extraction time at 1600 rpm, 30 mL sample volume, sample solution pH 0.5, 200 µL methanol as desorption solvent (5 min sonication assisted) and 5% w/v NaCl. The LODs (3 S/N) calculated for 4-HB and 3,4-DHB were 0.08 and 0.14 µg/g, respectively. Good relative recoveries (72.6–110.6%) and reproducibility values (RSD < 8.5%, $n = 9$) were obtained. The developed G-Fe_3O_4 MSPE method offered is simple, easy, environmental friendly and efficient for extraction of the two phenolic acids from stingless bee honey samples.

1. Introduction

The indigenous stingless bee species which originated from the tribe of Meliponini have populated the earth for over 65 million years as evident by the oldest known bee fossil, *Trigonaprisca* from the late Cretaceous New Jersey (de Camargo & de Menezes Pedro, 1992). About 700 species have been documented mainly in tropical regions (Mohd, Sajap, Rosliza, & Suri, 2010) and classified into several genera including *Dectylurina, Melipona, Meliponula, Lestrimelitta and Trigona.* Among the genera, *Trigona* has become the largest genus of stingless bees found exclusively in the tropical regions.

Apart from their role as pollinators in crops production, stingless bees produce highly beneficial products which include honey, beeswax, pollen, propolis, royal jelly, bee brood and bee venom (Bradbear, 2009). The most common product is honey which has been widely consumed by human due to its great taste and high medicinal value. Stingless bee honey exerts unique properties such as having unusual degree of acidity, sweetness and sourness. The composition of stingless bee honey includes the mixture of carbohydrates, with fructose and glucose as the major constituents, water and pharmacologically active compounds (Jaapar, Jajuli, & Mispan, 2016).

Several research have proved their ability to demonstrate pharmacological activities such as antibacterial (Massaro, Shelley, Heard, & Brooks, 2014), antioxidant (Roowi et al., 2012) and anticataract (Vit, 2002). The pharmacological properties of honey are highly correlated with the composition of the phenolic compounds (Rao, Krishnan, Salleh, & Gan, 2016) which also contribute to its flavour, colour and taste. Honey is rich in phenolic compounds mainly flavonoids and phenolic acids which are widely known by their capability as potent antioxidants. Phenolic acids are the secondary metabolites having phenols that possess one carboxylic acid functionality. They may exist in free form, esterified or soluble esters and insoluble-bound form (Shahidi & Yeo, 2016). There are two classes of phenolic acids which are hydroxybenzoic and hydroxycinnamic acid with high amount in bound form. However, the free phenolic acids are more easily absorbed into the body (Roowi et al., 2012). Although there is no specific

Abbreviations: 4-HB, 4-Hydroxybenzoic acid; 3,4-DHB, 3,4-Dihydroxybenzoic acid; NH_4OH, Ammonia solution; BET, Brunauer, Emmet and Teller; FTIR, Fourier transform infrared spectroscopy; G-Fe_3O_4, Graphene-magnetite; HPLC-UV/Vis, High performance liquid chromatography with ultravioletvisible detection; $FeCl_36H_2O$, Iron(II) chloride tetrahydrate ($FeCl_24H_2O$) and iron(III) chloride hexahydrate; LVSEM, Low vacuum scanning electron microscope; MSPE, Magnetic solid phase extraction; MNPs, Magnetic nanoparticles; Fe_3O_4, Magnetite; NaCl, Sodium chloride; TGA, Thermogravimetric analysis; TEM, Transmission electron microscope; VSM, Vibrating sample magnetometry; XRD, X-ray diffraction spectroscopy

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legislation of these compounds in food, Malaysian labelling regulation requires that nutrient and health claims should be based on scientific findings (Ministry of Health Malaysia, 2010). However, the constituents of phenolic acids are only in small amount which made their analysis become a great challenge. Therefore, searching for the most suitable extraction, pre-concentration and analysis methods for phenolic acids have emerged with several factors to be taken into considerations including their efficiency, selectivity, sensitivity and most importantly environmental friendly.

Several methods have been demonstrated for the extraction of phenolic acids such as 4-hydroxybenzoic, 3,4-dihydroxybenzoic, vanillic, gallic, *p*-coumaric and syringic acids from various matrices. These include supercritical fluid extraction (Ghafoor, AL-Juhaimi, & Choi, 2012), ultrasound-assisted extraction (Park, Tang, & Row, 2014) and solid-phase extraction (SPE) (Alarcón Flores, Romero-González, Garrido Frenich, & Martinez Vidal, 2012; Ahmed, Obbed, Wabaidur, Alothman, & Al-Shaalan, 2014). SPE is the most common method used in various applications. However, using SPE for the extraction of phenolic acids gave some drawbacks that include high cost due to the usage of cartridge, high sample volume as no agitation forces applied to enhance the adsorption and time-consuming. Therefore, modification of the SPE technique through the introduction of magnetic nanoparticles (MNPs) as adsorbent has been established to propose a simpler and efficient method known as magnetic solid phase extraction (MSPE).

However, MNPs such as magnetite promote some limitations due to its aggregation, low stability in acidic medium and is easily oxidized. These have led to their modifications and developments to enhance the selectivity of the modified adsorbent towards the target analytes and improve their efficiency. Over the years, several developments on the MSPE adsorbents have been reported. This include through the functionalization of the MNPs using chrysin (Abd Ali, Wan Ibrahim, Sulaiman, Kamboh, & Sanagi, 2016), silica-based (Nodeh, Wan Ibrahim, Kamboh, & Sanagi, 2017), ionic liquids (Liu, Li, Takafuji, Ihara, & Qiu, 2017), molecularly imprinted polymer (Bagheri, Molaei, Asgharinezhad, Ebrahimzadeh, & Shamsipur, 2016) and many more. Besides, using graphene as adsorbent had become a trend. Many research have reported the development of adsorbent using graphene-based materials in various matrices including biological fluids (Hashemi, Bagheri, Afkhami, Amidi, & Madrakian, 2018), water (Nodeh et al., 2017) and food samples (Zhang, Wang, Hao, Shi, & Wang, 2016). Graphene has high surface area of theoretically 2630 m^2/g (Stoller, Park, Yanwu, An, & Ruoff, 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). Due to these advantages, this study was aimed to synthesize and apply G-Fe_3O_4 adsorbent coupled with high performance liquid chromatography with ultraviolet–visible detection (HPLC-UV/Vis) for the determination of 4-hydroxybenzoic acid (4-HB) and 3,4-dihydroxybenzoic acid (3,4-DHB) in stingless bee honey. The physico-chemical characteristics of the sorbent were investigated. The MSPE method was validated and applied for extraction of the targeted phenolic acids in five honey samples.

2. Materials and methods

2.1. Standards and reagents

4-HB (purity ≥ 98%), 3,4-DHB (purity ≥ 96%), sodium hydroxide pellet and potassium dihydrogen phosphate (both purity ≥ 99%), iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$) (purity ≥ 98%) and iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) (purity ≥ 99%) were purchased from Merck (Darmstadt, Germany). Isopropanol was obtained from J.T. Baker (Pennsylvania, USA), while HPLC grade acetonitrile and methanol, hydrochloric acid (37%) and ammonia solution (28%) (NH_4OH) were supplied by QReC (Selangor, Malaysia). Sodium chloride (NaCl) and commercial iron (II, III) oxide magnetic (Fe_3O_4) were purchased from Bendonsen Laboratory Chemicals (Bendonsen, Norway). Ultrapure water (18.2 MΩ) was obtained from a Milli-Q Gradient water system from Millipore (Massachusetts, USA).

2.2. Instrumentations

Fourier transform infrared spectroscopy (FTIR) spectra were recorded using a Perkin Elmer 1600 Series FTIR Spectrometer (Massachusetts, USA) in the transmission range of 400–4000 cm^{-1}. Crystal structure of the synthesized materials was studied using X-ray diffraction (XRD) (Rigaku, Japan). Thermogravimetric analysis (TGA) was carried out using a Pyris Diamond thermogravimetric analyser from PerkinElmer (Yokohama, Japan) at a rate of 20 °C/min from 30 to 800 °C. Vibrating sample magnetometry (VSM) analysis was performed using a LAKESHORE 7404 series from Lake Shore Cryotronics, Inc (Westerville OH, USA). VSM was applied for monitoring the magnetic properties of the adsorbent at room temperature with maximum field (15000G). Surface morphology was observed using a JSM-6390 low vacuum scanning electron microscope (LVSEM) and JEM-2100 transmission electron microscope (TEM) from JEOL (Tokyo, Japan). Nitrogen adsorption analysis was performed on a 3 Flex Surface Characterization Analyzer from Micrometrics Instrument Corporation (Georgia, USA) using nitrogen gas as adsorbate at 77 K. The HPLC system was equipped with a JASCO PU-2080 pump (Tokyo, Japan), a Shimadzu SPD-10A UV/Vis detector (Tokyo, Japan) and PowerChrom software from eDAQ Pty. Ltd. (NSW, Australia). Chromatographic separation was performed using an Eclipse Plus C_{18} column (5 μm, 4.6 × 100 mm) from Agilent Technologies (Delaware, USA) and mobile phase of methanol:phosphate buffer (pH 2) (30:70% v/v) at a flow rate of 1.0 mL/min. The UV/Vis detector wavelength was set to 260 nm. Sample injection volume was 10 μL using a syringe from Agilent Technologies (Delaware, USA) into a Rheodyne injector.

2.3. Synthesis of graphene-magnetite adsorbent

Graphene-magnetite (G-Fe_3O_4) was prepared in a single-step procedure adapted from a previous work (Nodeh, 2015). First, 0.7 g $FeCl_2{\cdot}4H_2O$ and 1.35 g $FeCl_3{\cdot}6H_2O$ were mixed in ultrapure water followed by the addition of 0.5 g graphene. The final volume of the mixture was made up to 250 mL using ultrapure water and stirred until a homogenous mixture was observed. The mixture was then heated at 40 °C whilst stirring and 12 mL NH_4OH (28%) was added drop wise into the mixture. The reaction was continued for 2 h followed by subsequent filtration and washing three times with ultrapure water. Finally, the product obtained was oven-dried for 24 h.

2.4. Magnetic solid phase extraction

G-Fe_3O_4 adsorbent (50 mg) was added to a sample solution adjusted to pH 0.5. Extraction process was conducted at room temperature and assisted using a ZX3 Advanced Vortex Mixer from VELP Scientifica (Usmate, Italy) for 5 min at 1600 rpm rotational speed. Then, separation of the adsorbed analytes from sample solution was aided using an external magnet. The liquid phase was decanted followed by ultrapure water washing (3 × 1 mL) to remove the remaining matrices. The analytes were then eluted by dispersion of the adsorbent in 200 μL MeOH assisted by ultrasonication using a Bransonic CPX3800H sonicator (Danbury, USA) for 5 min. Next, the eluate was isolated from the adsorbent using external magnet and collected in a 1.5 mL centrifuge tube. Finally, the eluate was injected into HPLC-UV/Vis instrument for the analysis of 4-HB and 3,4-DHB.

2.5. Preparation of honey sample and standard solutions

Five stingless bee honey samples originated from the *Trigona* spp.