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Analytical Methods

Phenolic acid analysis and antioxidant activity assessment of oil palm (*E. guineensis*) fruit extracts

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ABSTRACT

Phenolic compounds in oil palm fruit (*E. guineensis*) were extracted in soluble free (SFP), insoluble-bound (ISBP) and esterified (EFP) forms. The total phenolic content (TPC) of the oil palm fruit extracts was determined using the Folin–Ciocalteu method and found to range from 5.03 to 9.04 g/L per g of dried weight (DW). The antioxidant activities of oil palm phenolic extracts were analysed using free radical scavenging assays and results showed that oil palm phenolic extracts contained antioxidant activities in the order of ISBP > EFP > SFP. Eight different phenolic acids were identified and quantified using a simple reversed-phase high performance liquid chromatography (HPLC) with a diode array detector (DAD) and liquid chromatography/tandem mass spectrometry (LC/MS/MS). Ferulic, *p*-hydroxybenzoic and *p*-coumaric acid were the dominant phenolic acids found in oil palm fruit extracts and ranged from 55 to 376 μg/g of DW.

1. Introduction

There is general consensus that free radical induced oxidative stress damage biomolecules in the human body and lead to degenerative diseases such as coronary heart disease and cancer (Arts, Dallinga, Voss, Haenen, & Bast, 2003). Free radical reactions can also induce the deterioration of food quality, nutrition losses, rancidity and complex chemical changes in food lipids (Fukumoto & Mazza, 2000). Various antioxidants have been used to increase the stability of food products. However, there is growing concern on the use of synthetic antioxidants and this has prompted studies on natural antioxidants such as phenolic compounds.

Phenolic compounds as the natural compounds found in plants comprise groups like the phenolic acids, flavonoids and tannins. Phenolic acids are hydroxy derivatives of aromatic carboxylic acids, which arise from either the benzoic acid or the cinnamic acid group. They differ according to the number and position of hydroxylation and methoxylation of the aromatic ring. Gallic, protocatechuic, *p*-hydroxybenzoic and vanillic acids are the derivatives of benzoic acid, whilst caffeic, *p*-coumaric and ferulic acids are the derivatives of cinnamic acid. Epidemiological data suggest that phenolic acids have strong inhibitory activity on oxidation induced by peroxyl radicals (Hu & Kitts, 2001) and accordingly have attracted special attention lately.

There are numerous publications on the antioxidative activities of plant-derived phenolics (Liu et al., 2002; Materska & Perucka, 2005; Wang & Ballington, 2007). Antioxidant capacity of a substrate can be measured through the ability of the substrate to intercept free radicals by scavenging or trapping methods (Huang, Ou, & Prior, 2005). Huang et al. (2005) stated that single electron transfer (ET) assays measure the colour changes of an oxidant due to reduction by an antioxidant. The extent of the colour changes is correlated with the sample's antioxidant concentrations.

Like all fruits, the oil palm fruit is a rich source of water-soluble phenolic antioxidants. These antioxidants are discarded along with the aqueous waste stream during the milling process to extract palm oil from oil palm fruits. A novel process for the extraction of palm phenolics from this aqueous stream has been patented (US Patent, 7387802) and these palm phenolics have been found to possess health benefiting properties (Sundram, Sambanthamurthi, & Tan, 2003). Instead of investigating phenolics extracted from the vegetation liquor produced during palm oil milling, a later study was carried out to extract, analyse and quantify soluble free (SFP), insoluble-bound (ISBP) and esterified (EFP) phenolic compounds directly extracted from oil palm fruits (Neo, Azis, Tan, & Tan, 2008).

In this study, we present a high performance liquid chromatography (HPLC) technique coupled with LC–MS–MS confirmation for identification of phenolic acids in SFB, ISFB and EFB. In order to determine the antioxidative potential of oil palm phenolics, the

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antioxidant activities of the oil palm phenolic extracts were also analysed using radical scavenging assays like 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay and ferric ion reducing antioxidant parameter (FRAP) assay in this study.

2. Materials and methods

2.1. Chemicals

Acetone, hydrochloric acid (HCl), diethyl ether, ethyl acetate and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Pittsburgh, PA). Methanol, *n*-hexane, anhydrous sodium sulphate, sodium carbonate, Folin–Ciocalteau reagent, acetonitrile, glacial acetic acid, sodium acetate and ferric chloride ($FeCl_3{\cdot}6H_2O$) were obtained from Merck (Darmstadt, Germany). Gallic acid ($C_7H_6O_5$, M_W = 170.12, >98% pure), protocatechuic acid ($C_7H_6O_4$, M_W = 154.12, >97% pure), *p*-hydroxybenzoic acid ($C_7H_6O_3$, M_W = 138.12, >99% pure), caffeic acid ($C_9H_8O_4$, M_W = 180.16, >98% pure), vanillic acid ($C_8H_8O_4$, M_W = 168.15, >97% pure), syringic acid ($C_9H_{10}O_5$, M_W = 198.17, >95% pure), *p*-coumaric acid ($C_9H_8O_3$, M_W = 164.16, >98% pure), ferulic acid ($C_{10}H_{10}O_4$, M_W = 194.18, 99% pure), DPPH radical, ABTS radical, 2,4,6-*tris*-2,4,6-tripyridyl-2-triazine (TPTZ), potassium persulphate and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). All reagents used were either analytical or HPLC grade. Milli-Q water (18.2 MΩ cm) was used in all experiments. Spectrophotometric analyses were performed using a Perkin–Elmer's Lambda 12 UV–visible spectrophotometer (Waltham, MA, USA). Quantification of phenolic acids was conducted using a Perkin–Elmer high performance liquid chromatography (HPLC) (Waltham, MA, USA) 200 series equipped with a diode array detector (DAD). Liquid chromatography/tandem mass spectrometry (LC/MS/MS) for further identification of the phenolic acids was performed using Applied Biosystem Q-trap 4000 (Foster City, CA) multiple ion trap mass spectrometer equipped with an Agilent 1100 series liquid chromatography system (Wilmington, DE).

2.2. Preparation of oil palm fruit sample

Oil palm inflorescences with flowers ready for pollination were taken as the first week of ripening. Inflorescence with flowers ready for pollination exudes a sweet smell similar to that of aniseed and is pale yellow in colour. The flowers turn red 2–3 days after being pollinated. Ripe fruits of *E. guineensis* (22 weeks) used in this study were freshly harvested from a plot in an oil palm plantation located at Bangi, Malaysia during the dry season. The fruits were collected from five different fruit bunches, composited into a single sample and divided into four lots for further analysis. The mesocarp of each fresh sample was manually cut into small pieces using a small knife and deoiled by Soxhlet extraction with *n*-hexane.

2.3. Isolation of soluble free (SFP) phenolic compounds

Isolation of SFP was conducted as described by Neo et al. (2008). One gram of deoiled mesocarp was weighed and homogenised with 70% aqueous methanol/70% aqueous acetone (1:1, v/v) at room temperature. Each homogenised mixture was centrifuged and the supernatants were pooled and evaporated under vacuum to a smaller volume. The aqueous suspension was adjusted to pH 2 and centrifuged to bring down the cloudy precipitate. The cloudy precipitate was set aside for extraction of esterified phenolic acids. The aqueous phase was extracted with hexane to remove free fatty acids and other lipid contaminants. The free phenolics in the aqueous phase were extracted with diethyl ether–ethyl acetate (DE–EA) (1:1, v/v) with a solvent to aqueous phase ratio of 1:1 (v/v). The DE–EA extracts were pooled and treated with anhydrous sodium sulphate to remove moisture, filtered, evaporated to dryness under vacuum at 45 °C and re-dissolved with 5 mL of methanol. The aqueous extract was pooled with the cloudy precipitate for subsequent extraction of esterified phenolics.

2.4. Isolation of esterified (EFP) phenolic compounds

Isolation of EFP was conducted as described by Neo et al. (2008). The esters were hydrolysed directly with NaOH (4 N) under a stream of nitrogen at room temperature. The hydrolysed solution was then adjusted to pH 2, and hexane was added to remove residual oil. The pH adjusted extract was then washed with DE–EA mixture. The extracts were combined, evaporated to dryness under vacuum leaving the esterified phenolic compounds, which were then re-dissolved with methanol to a final volume of 5 mL.

2.5. Isolation of insoluble-bound (ISBP) phenolic compounds

Isolation of ISBP was conducted as described by Neo et al. (2008). The deoiled mesocarp after the methanol–acetone extractions was hydrolysed with 4 N NaOH under nitrogen at room temperature. After acidification at pH 2 and centrifugation, the clear supernatant was extracted with hexane and then with DE–EA and re-dissolved with methanol to a final volume of 5 mL.

2.6. Determination of total phenolic content (TPC)

The TPC of the extracts was estimated colometrically using the Folin–Ciocalteu method (Waterhouse, 2002). Absorbance was measured at 765 nm with gallic acid as reference standard and methanol as blank. Results were expressed as GAE (Gallic Acid Equivalent). The gallic acid calibration curve was established from 50 to 250 mg/L.

2.7. Determination of radical scavenging abilities of palm phenolic compounds

The DPPH, ABTS and FRAP assays were performed according to the method of Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, and Byrne (2006). The DPPH stock solutions was prepared and diluted to an absorbance of 1.10 ± 0.03 U at 515 nm. The absorbance of the mixture of the extract and DPPH working solutions was measured at 515 nm and the scavenging activity expressed as μg Trolox equivalents (TE).

$ABTS^{\cdot+}$ was prepared by mixing an ABTS stock solution (7.4 mM) with 2.6 mM potassium persulphate at a ratio of 1:1 (v/v) and diluted to obtain an absorbance of 1.10 ± 0.03 U at 734 nm. Fresh $ABTS^{\cdot+}$ solutions were prepared for each assay. The absorbance of the mixture of the extract and $ABTS^{\cdot+}$ working solutions was then measured at 734 nm and the scavenging activity expressed as μg TE.

FRAP reagent was prepared daily by mixing 300 mM acetate buffer (pH 3.6 with acetic acid) and 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3{\cdot}6H_2O$ in a ratio of 10:1:1 (v/v). The FRAP reagent was warmed to 37 °C before use. The absorbance of the mixture of the extract and the FRAP reagent was measured at 593 nm and the scavenging activity expressed as μg TE.

2.8. Purification of phenolic acids

Phenolic acids in the oil palm fruit extracts (SFP, EFP, and ISBP) were isolated using solid phase extraction (SPE) technique. The ex-