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Analytical Methods

Development of a reversed-phase high performance liquid chromatography (RP-HPLC) procedure for the simultaneous determination of phenolic compounds in peanut skin extracts

Maria Leonora dL. Francisco, A.V.A. Resurreccion *

Department of Food Science and Technology, The University of Georgia, 1109 Experiment Street, Griffin, GA 30223-1797, USA

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ABSTRACT

A reversed-phase high performance liquid chromatography (RP-HPLC) procedure was developed for simultaneous determination of five phenolic acids, two stilbenes and eight flavonoids in peanut skin extract. A C_{18} column fitted with diode array detection at 250 and 320, 280 and 370, and 306 nm for phenolic acids, flavonoids and stilbenes, respectively, with mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in 100% acetonitrile. Phenolic compounds were eluted with good resolution (Rs > 1.5) within 95 min as follows: gallic, protocatechuic, epigallocatechin, catechin, β-resorcylic (internal standard), caffeic, procyanidin B_2, epicatechin, epigallocatechin gallate, *p*-coumaric, ferulic, piceid, epicatechin gallate, catechin gallate, resveratrol and quercetin. The variation in recovery and reproducibility in peak area was <11 and <2.5%, respectively. The correlation coefficients, *r*, of calibration curves of the 15 compounds were >0.999. The method was used to quantify phenolic compounds in peanut skin extracts from Runner, Virginia and Spanish peanuts.

1. Introduction

A report identified peanut skins as one of the important sources of antioxidants with healthcare application that food manufacturer's can tap into (http://www.kaloramainformation.com/Healthcare-Opportunities-Food-1468551/). Peanut skins have a pink-red colour and an astringent mouth feel when consumed. They are typically removed before peanut consumption or inclusion in confectionary and snack products. In the early 1940's, these were initially thought to be toxic. But after a thorough examination by Dr. Jack Masquelier, then a doctoral candidate at the Faculty of Medicine and Pharmacy, University of Bordeaux, in France, peanut skin was found nontoxic, and protects and strengthens blood vessels (Louis, 1999). The colourless extract obtained was named OPC, oligomer proanthocyanidins. Peanut skins are rich in phenolic compounds and potentially other health-promoting compounds. Seventeen percent by weight of peanut skins are proanthocyanidins, consisting of low and high molecular weight oligomers (Karchesy & Hemingway, 1986). Lou et al. (1999) made a comprehensive analysis of the water-soluble phenolic extract from peanut skins, resulting in six A-type proanthocyanidins, including procyanidins A_1 and A_2, and three newly found epicatechin oligomers. They isolated ten compounds from the water-soluble fraction of peanut skins, including eight flavonoids and two novel indole alkaloids, reported for the first time from a natural source (Lou, Yuan, Yamazaki, Sasaki, & Oka, 2001). In 2004, they isolated five oligomeric proanthocyanidins, B_2, B_3 and B_4 from the water-soluble fraction and two new polyphenols, epicatechin-(2-β-*O*-7,4-β-6)-[epicatechin-(4-β-8)]-catechin and epicatechin-(2-β-*O*-7,4-β-8)-[epicatechin-(4-β-8)]-catechin-(4-α)-epicatechin, based on their spectral data (Lou et al., 2004). Huang, Yen, Chang, Yen, and Duh (2003) isolated and identified the ethanolic extract fraction from peanut seed testa that showed the highest yield and marked antioxidant activity. Thin layer chromatographic separation of this fraction allowed the isolation of the antioxidant component in peanut seed testa which was identified as ethyl protocatechuate (3,4-dihydroxybenzoic acid ethyl ester). Other types of compounds isolated and identified in peanut skins include phenolic acids, flavonoids and stilbenes (Yu, Ahmedna, & Goktepe, 2005). Yu, Ahmedna, Goktepe, and Dai (2006) identified and quantified catechins, procyanidin dimers, trimers and tetramers using reversed-phase high performance liquid chromatography (RP-HPLC). These compounds were detected in chemically purified peanut skin aqueous and ethanol extracts. Furthermore, higher concentrations of compounds mentioned were observed in raw peanut skins than roasted peanut skins. Caffeic acid, chlorogenic acid, ellagic acid and resveratrol were identified but not quantified due to very small

* Corresponding author. Tel.: +1 770 412 4736; fax: +1 770 412 4748.
E-mail address: aresurr@uga.edu (A.V.A. Resurreccion).

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peaks, as a result of suppression by major procyanidin peaks. Resveratrol was found to be present at higher levels in the seedcoat than the peanut kernel. Seed coats from Runner and Virginia types contained approximately 0.65 μg/g of seed coat, which is equivalent to <0.04 μg/seed (Sanders, McMichael, & Hendrix, 2000). Ethanolic extract prepared from defatted peanut skins even showed higher resveratrol contents. The ethanolic extract contained 91.4 μg/g while the dry peanut skins contain 9.07 μg/g (Nepote, Grosso, & Guzman, 2004).

Reverse-phase chromatography is the most popular mode of analytical liquid chromatography for phenolic compounds. A RP-HPLC method for food phenolics requires optimising a wide variety of mobile-phase conditions (ionic strength, pH, ion pair, organic modifier, etc.) and column parameters (Lee, 2000). Columns range from 100 to 300 mm in length and usually with a 4.6 mm internal diameter. The most often used columns have been packed with C_{18} column material. Elution systems are usually binary with an aqueous acidified polar solvent such as aqueous acetic acid, perchloric acid, phosphoric acid, or formic acid (solvent A) and a less polar organic solvent such as methanol or acetonitrile, possibly acidified (solvent B). The greatest alteration observed in the mobile phase was the type of acid used as the modifier to minimise peak tailing (Merken & Beecher, 2000). The pH range most often used for RP-HPLC for phenolics is low, between 2 and 4. The pH and ionic strength of the mobile phase are known to influence the retention of phenolics on the column, depending on whether there is protonation, dissociation, or a partial dissociation (Lee, 2000). HPLC runs are generally an hour maximum, with equilibration between runs (Merken & Beecher, 2000).

Phenols absorb in the ultraviolet (UV) region. Most of the phenolic acids displayed their maxima at 246–262 nm with a shoulder at 290–315 nm (Lee, 2000). Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings A, B, and C. Two absorption bands are characteristic of flavonoids. Band II, with a maximum in the 240–285 nm range, is believed to arise from the A ring. Band I with a maximum in the 300–550 nm range, presumably arises from the B-ring (Merken & Beecher, 2000). Food phenolics are commonly detected using UV–vis and photodiode array (DAD) detectors.

Several hundred papers on the HPLC of phenolic compounds have been published in the past 20 years, yet HPLC of phenolic compounds can detect across one, two or perhaps three classes or subclasses in one analysis. Foods may contain several classes of phenolic compounds (Merken & Beecher, 2000). Phenolic extracts are always a mixture of different classes of phenolics that are soluble in the solvent system used (Naczk & Shahidi, 2004). Due to the large number, and the structural variations in closely related food phenolic compounds, analytical procedures for the analysis of individual phenolic compounds have been relatively difficult and complicated (Lee, 2000). The ideal profiling method should be as simple as possible, should detect all the compounds present, should provide as much information as possible for each peak in the chromatogram for the purpose of identification, structural evaluation, and quantification, and should accomplish all this in a single chromatographic run (Harnly, Bhagwat, & Lin, 2007). A review of some examples of profiling methods for the last 15 years is discussed by Harnly et al. (2007). With the increasing interest in peanut skins as a rich source of antioxidants, it is imperative to accurately determine and quantify as many phenolic compounds to answer the demand for timely information on health-promoting compounds present in peanut skins. The objective of this study was to develop a RP-HPLC method using DAD for the identification and quantification of phenolic compounds in peanut skin extracts.

2. Materials and methods

2.1. Chemicals and materials

Standards including gallic acid, quercetin, catechin, (−)-catechin gallate, (−)-epicatechin gallate, (−)-epigallocatechin, procyanidin B_2, and β-resorcylic acid (internal standard, IS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Resveratrol, (−)-epigallocatechin gallate, caffeic acid, *p*-coumaric acid, protocatechuic acid, ferulic acid, and (−)-epicatechin were purchased from Fisher Scientific (Atlanta, GA, USA). Methanol, acetonitrile and formic acid were purchased from VWR (West Chester, PA, USA).

2.1.1. Standard solutions for quantifying phenolic compounds

Stock solutions of gallic acid (1000 ppm), quercetin (1000 ppm), catechin (1000 ppm), (−)-catechin gallate (2000 ppm), (−)-epicatechin gallate (2000 ppm), (−)-epigallocatechin (2000 ppm), procyanidin B_2 (2000 ppm), β-resorcylic acid (1000 ppm), resveratrol (1000 ppm), (−)-epigallocatechin gallate (1000 ppm), caffeic acid (1000 ppm), *p*-coumaric acid (1000 ppm), protocatechuic acid (1000 ppm), ferulic acid (1000 ppm) and (−)-epicatechin (1000 ppm) were prepared by dissolving the compounds separately in methanol, and stored in 2 oz. amber bottles (VWR West Chester, PA, USA) at −15 °C.

2.2. Preparation of peanut skin extract

Raw peanuts (2004 crop year) were purchased from Golden Peanut Company (GA). Runner medium (Georgia Green) were harvested in Dawson, GA; Virginia medium in Aulander, NC; and No. 1 Spanish in Anadarko, TX. All peanut bags were stored for 1 month under refrigerated storage in a cold room at 4 °C prior to analysis. Raw, peanuts were manually sorted to remove foreign material or damaged kernels. Kernels were manually peeled and skins were packaged in 12.7 × 16.5 cm polyethylene bags (Koch Supplies, N. Kansas City, MO, USA) flushed with nitrogen gas (medical grade, Air Products and Chemicals, Inc., Allentown, PA, USA), immediately heat-sealed (Thermal Impulse Heat Sealer, Model 14C/CAB, Vertrod Corp., USA) then stored in a walk-in freezer at −15 °C until used in HPLC analyses.

The extraction of phenolic compounds in peanut skin was carried out according to the method published by Nepote, Grosso, and Guzman (2005). Briefly, 20 mL of 70% ethanol was added to a flask containing 1 g of peanut skins. The flasks were shaken for 10 min using a Wrist Action Shaker (Model 75, Burrell Corp., PA, USA) at ambient temperature. The extract was filtered through a Whatman no. 1 filter paper and the residue was extracted again under the same conditions, for a total of three extractions.

All flasks were wrapped with aluminium foil to prevent light degradation during extraction as done by Yu et al. (2005). The combined filtrate was transferred into an evaporating flask and placed in a water bath (HB4 basic, IKA, Fisher Scientific Co., Fair Lawn, NJ, USA) set at 40 °C according to Duh and Yen (1997) for drying extracts from peanut hulls. The extract was evaporated under vacuum for 15 min in a rotary evaporator (RV05 Basic 1B, IKA, Fisher Scientific Co., Fair Lawn, NJ, USA) to a concentrated extract of about 5 mL. The extract was transferred to a plastic cup (No. P325, Solo Cup Co., Urbana, IL, USA) and the evaporating flask was washed with approximately 2 mL of deionised water which was combined with the extract in the cup. Sample cups were sealed with aluminium foil and frozen overnight in a walk-in freezer at −15 °C. The frozen mixture was then freeze-dried (Virtis Genesis 25ES, SP Industries Inc., Gardiner, NY, USA) at <100 mTorr vacuum for 24 h. Dried extracts were scraped from the cups and packaged in