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Contributed Paper

Method Development and Determination of Phenolic Compounds in Broccoli Seeds Samples

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method with photo-diode array detection has been developed for the simultaneous determination of the main phenolics classes including, catechin, epicatechin, epigallocatechin gallate, gallic acid, quercetin and rutin. Phenolic compounds were separated within 35 min, using an octadecylsilyl column and a gradient elution system of acetonitrile-acetic acid solution (pH 3.0)-methanol as the mobile phase with a flow rate of 1.0 mL/min. The detection limits and quantitation limits of these compounds were in the range of 0.15-0.46 and 0.42-2.47 μg/mL, respectively. The recovery tests of all compounds range of 96–103% were obtained. This method was applied to the analysis of phenolic compounds in broccoli seeds genotypes cultivated in Thailand.

Keywords: catechin, epicatechin, epigallocatechin gallate, gallic acid, quercetin, rutin.

1. INTRODUCTION

Numerous reports pointed out that vegetables and fruits have excellent antioxidant properties, mainly of their polyphenolic constituents. The polyphenolic compounds (Figure 1) are comprised basically of phenolic acids, including benzoate and hydroxycinnamate derivatives, and flavonoids. Accordingly, flavanols and flavanol oligomers have been established to possess powerful antioxidant properties and other beneficial biological activities. The documents from animal and human studies, so far, indicated that flavanol monomers, including catechin, epicatechin, epigallocatechin gallate, were considered as potent anticarcinogens and/or antiatherogenic agents [1-8]. There is thus a constant need for isolation, examination and implementation of natural antioxidants. This present study was undertaken to develop an analytical procedure for polyphenolic composition of broccoli seeds originating cultivated in Thailand to provide information related to their antioxidant characteristics, which may be provided both quantitative assay and nutritional interest.

2. MATERIALS AND METHODS

2.1. Standards and Reagents

Gallic acid, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate (EGCG), quercetin and rutin were acquired from Sigma Chemical Co (St. Louis, MO). Acetic acid (glacial) was obtained from J.T. Baker (NJ). Methanol (MeOH) and acetonitrile (ACN) of HPLC grade solvents were purchased from Fisher (Milan, Italy).

In all cases, the water used was of HPLC quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA). The standard solution containing about 50 μg/mL of each of

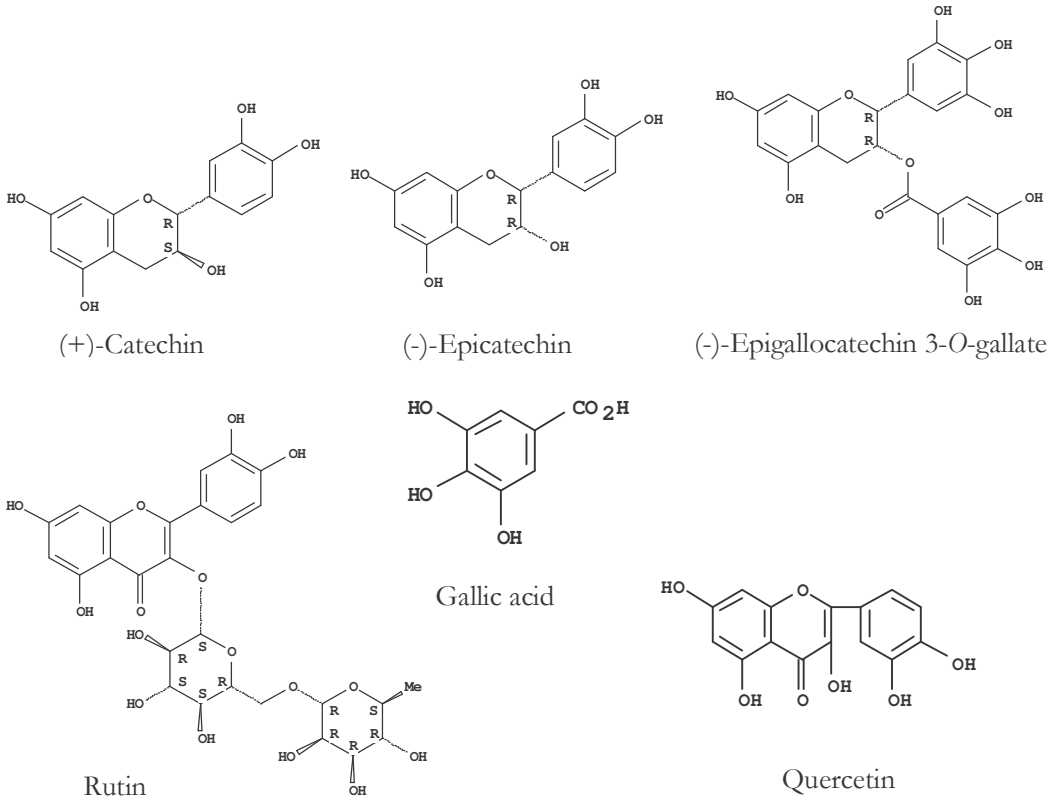

Figure 1. Chemical structures of the polyphenolic reference standards used in this study.

catechin, epicatechin, epigallocatechin gallate, gallic acid, quercetin and rutin was prepared in methanol and used in the method validation and analysis of broccoli seeds samples. All standards were stored in darkness at 5 ˚C.

2.2 Samples Preparation

Broccoli seeds were obtained from Phu Ruea Highland Cultivation Experimental Station, Loei province, Thailand. There were five broccoli seeds cultivars which included 'Green Queen', 'Packman', 'Pak Ging', 'Rod Fai', and 'Top Green #067'. The seeds were harvested in April-May, 2003 and were packed then sealed in aluminium foil laminated sachets. The seeds were stored unopened and refrigerated at 4 ˚C until use.

Approximately 10 g of the seeds were ground with a pestle and a mortar, and then transferred to a centrifuge tube with 10 mL of aqueous methanol (80%, v/v) in diluted HCl acid media (1%, v/v). The mixture was centrifuged at 5000 rpm for 20 min and the extraction procedure was repeated twice. The combined supernatant was then filtered through a 0.45 μm membrane filter before injection.

2.3 High-performance Liquid Chromatography: Instrument and Conditions

Chromatographic analyses were carried out on an HP 1090, series II, liquid chromatography apparatus, coupled to a HP 1090 diode array detector (Hewlett Packard, Wilmington, DE, USA). The separation was achieved on a Beckman Ultrasphere 5μm (4.6 x 150 mm) column at 30 ˚C. Columns were extensively equilibrated with a minimum of 100 column void volumes of solvents. The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). The system was run with the following gradient elution program: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30

min, 100%A. There was a 10 min post run at initial conditions for equilibration of the column. The flow rate was kept constant throughout the analysis at 1 mL/min. Injections were accomplished with a 10 μL fixed loop and the analysis was monitored at 280 nm. Prior to HPLC analysis, all solutions were filtered through 0.45 μm membranes filter (Sartorius, Germany) and then degassed in an ultrasonic bath for 30 min. Identification was based on retention times and on-line spectral data in comparison with authentic standards. Quantification was performed by establishing calibration curves for each compound determined, using the standards. The analytical data were performed using HP ChemStation data analysis software, version 6.03.

3. Results and Discussion

3.1 Method Development

In previously studies found that there was a direct relationship between antioxidant activity and total phenolic content in selected broccoli seeds. Phenolic compounds had a major contribution to antioxidant activity [4,7]. Among the many separation systems, the HPLC analysis include the use of a binary solvent system containing acidified water and a polar organic solvent were developed to specifically measure polyphenolic concentrations [9,10]. Initial trials assay, an elution program using phosphoric acid (0.1%), acetic acid solution (pH 3.0), acetonitrile and methanol were tested. Although acetic acid solution and methanol gave good resolution for the standard mixture, the system still could not separate several key polyphenolics in the standard mixture. Thus, several concentrations of acetonitrile (solvent A), acetic acid solution, pH 3.0 (solvent B) and methanol (solvent C) were evaluated. As methanol concentration was increased from 10 to 20%, retention factor (k) decreased significantly, with all of the phenolic compounds following a similar tendency. This resulted in the co-elution of most of the phenolic with isocratic elution using 20% methanol. Isocratic elution at lower concentrations such as 40% acetonitrile also resulted in poor chromatographic separation of (-)-epigallocatechin gallate, quercetin and rutin. This information was used to create an optimal gradient. The segmented gradient consisted of a shallow linear gradient from 5 to 10% acetonitrile, followed by isocratic elution of 20% acetonitrile for 10 min to improve the resolution of phenolic compounds eluting early; then the concentration of acetonitrile in the mobile phase was gradually raised to 100% to produce reasonable retention time for the components eluting later. The good separation was found with gradient elution program: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C; 30 min, 100%A. The resulting gradient program permitted resolution of all of the phenolic compounds within 35 min as represented in Figure 2. The wavelength between 210 and 360 nm is ordinarily used for the detection of polyphenolic compounds [1,9,10]. Then, 250, 280 and 320 nm wavelength were determined for the appropriate wavelength for the detection of all standard in this study. Each standard showed strong absorption at the wavelength 280 nm.

3.2 Method Validation

The validation of this newly developed analytical method was performed according to the ICH guidelines [11]. Detection limits, quantitation limits, linearity ranges, and recovery were evaluated using standard samples (Table 1). The limits of detection and quantification were calculated as singnal-to-noise ratios with nominal values of 3:1 and 10:1, respectively. Detection and quantitation limits of these compounds were in the range of 0.15-0.46 and 0.42-2.47 μg/mL, respectively. Linearity was evaluated on five calibration points (0.5, 1, 10, 50 and 100 μg/mL) with three measurements for each calibration point. All the analyses exhibited good linearity over the evaluated range with correlation coefficients between 0.996 and 1.000.

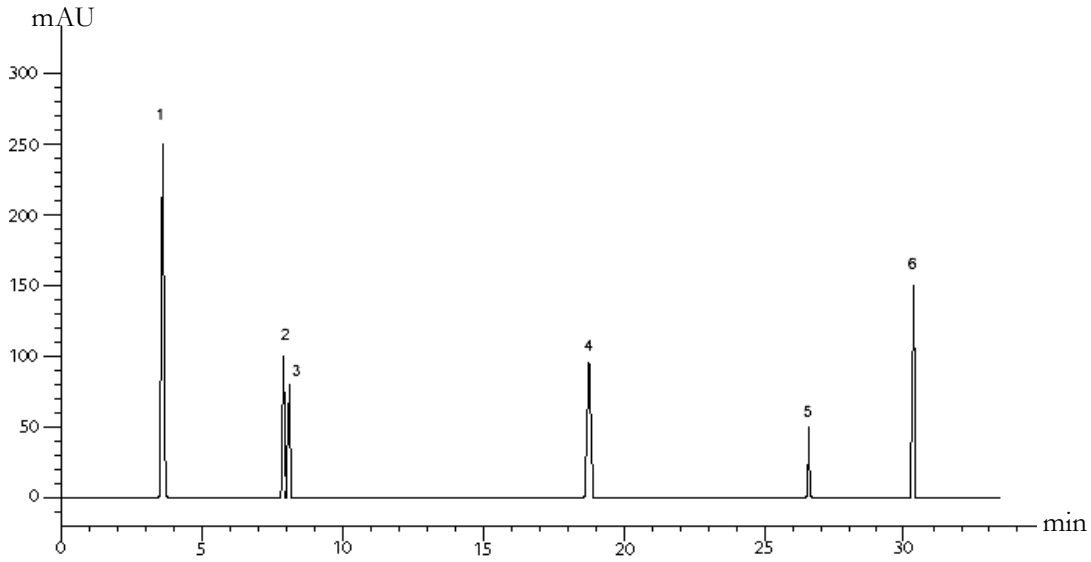

Figure 2. HPLC chromatogram of phenolic standards: 1, gallic acid (3.63 min); 2, catechin (7.92 min); 3, epicatechin (8.01 min); 4, (-)-epigallocatechin gallate (18.79 min); 5, quercetin (26.54 min) and 6, rutin (30.57 min).

Table 1. Parameters of calibration graphs for the phenolic standards in this study.

Peak no.	Phenolic compound	Linearity[a]	Detection limit (μg/mL)[b]	Quantitation limit (μg/mL)[b]	Recovery (%)[c]
1	Gallic acid	1.000	0.15	0.42	99.5 ± 2.0
2	(+)-Catechin	0.996	0.44	2.14	98.6 ± 3.4
3	(-)-Epicatechin	0.997	0.39	1.87	97.5 ± 3.6
4	(-)-EGCG	0.995	0.37	1.11	97.8 ± 2.6
5	Quercetin	0.997	0.46	2.47	100.5 ± 5.0
6	Rutin	0.998	0.21	1.18	104.6 ± 2.8

[a] Linearity was expressed as the correlation coefficient of each calibration curve, which was determined by five calibration points.
[b] Data were expressed as mean of triplicate measurements.
[c] Recovery are expressed as mean ± standard deviation carried out in broccoli seeds samples.

To demonstrate the method specificity, the performed peak purity tests were used and the photo-diode array analysis confirmed that each chromatographic peak of all standards was attributable to a single component (data not shown). In order to verify their accuracy, it was carried out recovery tests, adding known amounts of the standard sample to a preparation of broccoli seeds extract. In recovery assay, the samples were fortified with all standards at 10, 50 and 100 μg/mL. The recovery tests of all compounds range of 96–103%. To confirm the repeatability of the method, a preparation of seeds extract was analyzed repeatedly 3 separate times. The variation coefficients of all measurements were less than 1.2%. In all studies cases, purity peak was carefully examined by tracing spectra at up-slope, the apex and down-slope and a high elution purity was observed.

3.3 Analysis of Broccoli Seeds Samples

Preliminary separation and identification of individual phenolic compounds in broccoli seeds extract was conducted by HPLC. Sample peaks were identified by matching

against retention time of known phenolic standards under the same chromatography conditions. Due to the complexity and diversity of the natural mixtures of phenolic compounds in hundreds of extracts, then it have been reported that the same number of peaks (corresponding to the same retention times) were not observed in the chromatograms of the broccoli seeds extracts.

4. Conclusion

Using this analytical method, catechin, epicatechin, epigallocatechin gallate, gallic acid, quercetin and rutin could be determined simultaneously, and the validity of the method was also verified. There was no detectable phenolic compounds in broccoli seeds in all extracts from this study.

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