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Determination of Rutin, Catechin, Epicatechin, and Epicatechin Gallate in Buckwheat *Fagopyrum esculentum* Moench by Micro-High-Performance Liquid Chromatography with Electrochemical Detection

ANA-MARIA DANILA,[†,‡] AKIRA KOTANI,[‡] HIDEKI HAKAMATA,[‡] AND FUMIYO KUSU*[,‡]

Department of Analytical Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy "Gr.T.Popa", Iasi, Romania, and Department of Analytical Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan

A simple and sensitive method has been developed for determining rutin, catechin, epicatechin, and epicatechin gallate in buckwheat (*Fagopyrum esculentum* Moench) flour and seeds by micro-high-performance liquid chromatography with electrochemical detection. Chromatography was performed using an octadecylsilica column, acetonitrile−water−formic acid (13:87:1, v/v/v) as a mobile phase, and an applied potential at +0.5 V vs Ag/AgCl. We found that Japanese buckwheat flour contains rutin (12.7 mg/100 g), catechin (3.30 mg/100 g), epicatechin (20.5 mg/100 g), and epicatechin gallate (1.27 mg/100 g). The relative standard deviations for rutin, catechin, epicatechin, and epicatechin gallate peak heights were less than 0.86% ($n = 5$). The detection limit of rutin was 0.86 ng/mL. Moreover, the present method was applied to the distribution analysis of these compounds in buckwheat seed. The embryo proper and cotyledons of a mature buckwheat seed contained rutin with the highest concentration as compared to other parts. This method is useful in determining rutin, catechin, epicatechin, and epicatechin gallate in buckwheat with a small amount of sample for quality control in the food industry.

KEYWORDS: Rutin; catechin; epicatechin; epicatechin gallate; buckwheat; HPLC with electrochemical detection

INTRODUCTION

Common buckwheat (*Fagopyrum esculentum* Moench) is recognized as a healthy food in many countries (*1*) because it is rich in flavonoids, vitamins, amino acids, and other substances. One of the most important components of buckwheat is rutin, a flavonol glycoside compound, which has a hypotensive effect, antioxidant activities, and antagonizes the increase of capillary fragility associated with some hemorrhagic diseases (*2*).

The concentration of natural antioxidants may show strong variations depending on several factors including variety, location, and environmental conditions. Rutin contents in buckwheat grain varieties were reported to range from 12.6 to 35.9 mg/100 g dry weight (*3*).

Analytical methods suitable for measurement of rutin have mainly been based on reversed-phase high-performance liquid chromatography with diode array detection (HPLC-DAD) (*4*, *5*), reversed-flow micellar electrokinetic chromatography (RF-MEKC-UV) (*6*), capillary electrophoresis with ultraviolet detection (CE-UV) (*7*), capillary electrophoresis with electrochemical detection (CE-ECD) (*8*), and capillary zone electrophoresis with amperometric detection (CZE-AD) (*9*).

HPLC with electrochemical detection (HPLC-ECD) is a sensitive and selective method for the determination of redox compounds (*10*−*13*). To further improve the sensitivity, downsizing of the HPLC-ECD system such as using a microbore column is very useful, and we have succeeded in highly sensitive determination of flavonoids such as quercetin, hesperidin, baicalin, and baicalein by micro-HPLC-ECD (μHPLC-ECD) (*14*−*17*).

Therefore, a simple and sensitive method was developed for determining rutin, catechin, epicatechin, and epicatechin gallate in buckwheat flour and buckwheat seeds by μHPLC-ECD. Moreover, the present method was applied for determination of rutin, catechin, epicatechin, and epicatechin gallate concentrations in the bottom part of the endosperm, testa, the embryo proper and the cotyledons, and the endosperm of the buckwheat seeds. No articles about the rutin, catechin, epicatechin, and

* To whom correspondence should be addressed. Tel: +81-42-676-4549. Fax: +81-42-676-4570. E-mail: kusu@ps.toyaku.ac.jp.
† University of Medicine and Pharmacy "Gr.T.Popa".
‡ Tokyo University of Pharmacy and Life Sciences.

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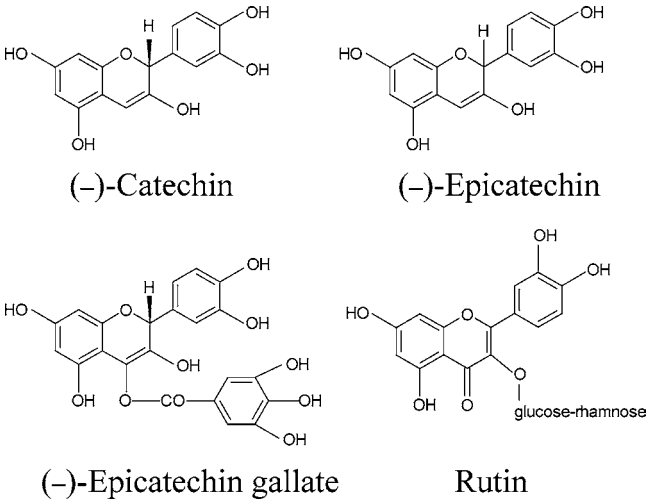

Figure 1. Structures of rutin, catechin, epicatechin, and epicatechin gallate.

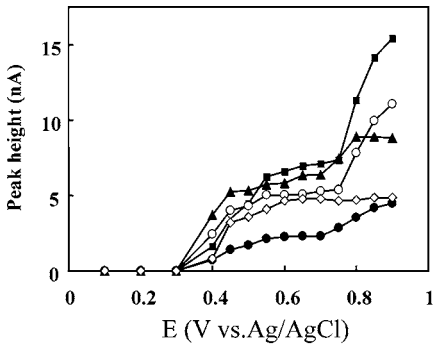

Figure 2. Hydrodynamic voltammograms of rutin (◇), catechin (■), epicatechin (○), epicatechin gallate (●), and ethyl gallate (▲) (IS).

epicatechin gallate distribution in different parts of buckwheat seed could be found in the literature.

MATERIALS AND METHODS

Chemicals. (−)-Catechin (>98%), (−)-epicatechin (>98%), and (−)-epicatechin gallate (>98%) (**Figure 1**) were purchased from Kurita Industrial (Tokyo, Japan). Rutin (>97%) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ethyl gallate (>98%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Commercial buckwheat flour was purchased from Togakusi Soba Honpo Co. Ltd. (Nagano, Japan). Buckwheat seeds were harvested in the middle of September 2005, in Shibetsu, Hokkaido, Japan. The hulls of the seeds were removed on May 26, 2006.

Apparatus. The μHPLC-ECD equipment was comprised of an LC-27A vacuum degasser (BAS, Tokyo, Japan), an LC-100 pump (BAS), a 7125 injector fitted with a 5 μL injection loop (Rheodyne, Cotati, CA), a 150 mm × 1.0 mm i.d., 3 μm Capcell Pak C_{18} UG 120 microbore ODS column (Shiseido, Tokyo, Japan), an FT-1 column oven (BAS), and an LC-4C electrochemical detector (BAS). A commercially available electrochemical cell (radial flow cell, BAS) was constructed from a glassy carbon working electrode, an Ag/AgCl reference electrode, and a stainless steel auxiliary electrode. A 5 μL sample solution or standard flavonoid solution was injected into the microbore ODS column maintained at 40 °C. Deaerated acetonitrile−water−formic acid (13:87:1, v/v/v) served as the mobile phase, at a flow rate of 40 μL/min. The detection potential for monitoring rutin, catechin, epicatechin, and epicatechin gallate was set at +0.5V vs Ag/AgCl. An internal standard (IS) method was used for the determination of rutin, catechin, epicatechin, and epicatechin gallate concentrations in the buckwheat flour and buckwheat seeds, and ethyl gallate was used as an IS.

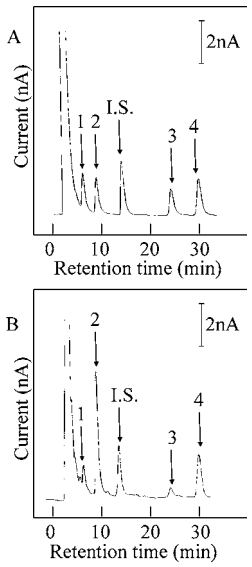

Figure 3. Chromatograms of rutin, catechin, epicatechin, epicatechin gallate, and ethyl gallate (IS) in (**A**) standard solution and (**B**) buckwheat flour obtained by μHPLC-ECD. Peaks: 1, catechin; 2, epicatechin, IS ethyl gallate (IS); 3, epicatechin gallate; and 4, rutin.

Table 1. Contents of Catechin, Epicatechin, Epicatechin Gallate, and Rutin in Buckwheat Flour and Recovery from Buckwheat Flour by μHPLC-ECD

	content ($n = 5$)		recovery ($n = 5$)		
compound	amount (mg/100 g)	RSD (%)	added amount (mg/100 g)	recovery (%)	RSD (%)
catechin	3.30	0.23	3.30	99.4	0.46
epicatechin	20.50	0.86	20.90	97.6	0.70
epicatechin gallate	1.27	0.52	1.27	92.3	0.86
rutin	12.70	0.68	12.65	90.1	0.76

Preparation of Buckwheat Flour Sample. Buckwheat flour (10 mg) was mixed with 1 mL of MeOH−H_2O (6:4, v/v). The sample was vortexed and sonicated for 30 s; these operations were performed three times and centrifuged at 3000 rpm for 5 min. Two hundred microliters of the supernatant was diluted with 800 μL of acetonitrile−water−formic acid (13:87:1, v/v/v) containing 75 ng/mL ethyl gallate (IS). The solution was filtered through a 0.45 μm membrane filter. A 5 μL volume sample solution or standard flavonoids were injected into the HPLC system.

Preparation of Buckwheat Seed Samples. The hull of buckwheat seed envelops the testa, endosperm, and embryo. The embryo and its two cotyledons are surrounded by endosperm, and they reach the testa only with their margins (*1*, *18*). The testa was removed by cutting with a small knife. The bottom part of the endosperm was removed from the seed groat, and then, the embryo and its two cotyledons were carefully separated from the endosperm. The cotyledons were hard and yellow-colored, while the endosperm was soft and white-colored, so they could be distinguished from each other. Every component was weighed on an analytical balance, and then, the same procedures were followed as described in the preparation of the buckwheat flour sample.

RESULTS AND DISCUSSION

Optimization of HPLC-ECD Conditions. Figure 2 shows hydrodynamic voltammograms of rutin, catechin, epicatechin, epicatechin gallate, and ethyl gallate. Rutin, catechin, epicatechin, epicatechin gallate, and ethyl gallate were oxidized at potentials more positive than +0.4 V vs Ag/AgCl. Two oxidation waves were observed in the hydrodynamic voltammograms of catechin, epicatechin, epicatechin gallate, and ethyl gallate, one at +0.5 V and the other at +0.8 V, while rutin

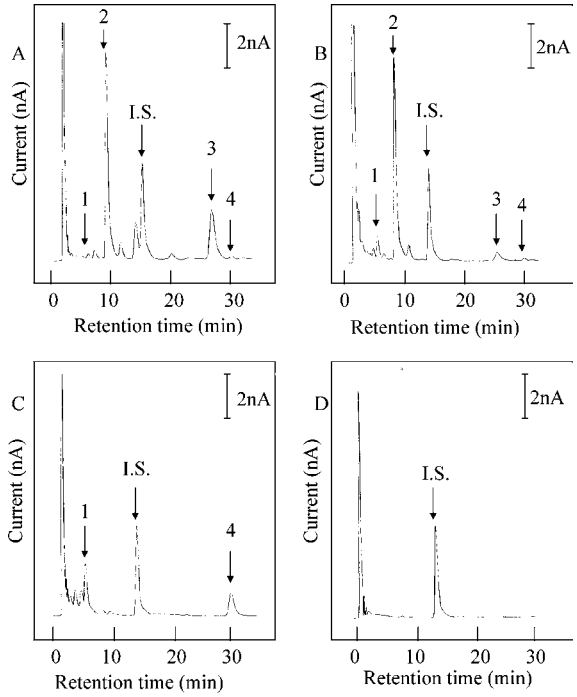

Figure 4. Chromatograms of rutin, catechin, epicatechin, and epicatechin gallate in (**A**) the bottom part of the endosperm, (**B**) testa, (**C**) embryo proper and cotyledons, and (**D**) endosperm in dehulled type I buckwheat seeds ($n = 5$ each). Peaks: 1, catechin; 2, epicatechin, IS ethyl gallate (IS); 3, epicatechin gallate; and 4, rutin.

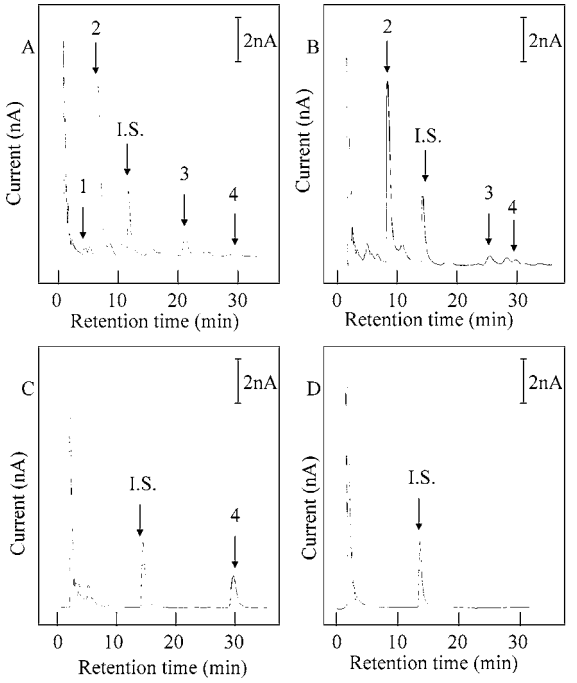

Figure 5. Chromatograms of rutin, catechin, epicatechin, and epicatechin gallate in (**A**) the bottom part of the endosperm, (**B**) testa, (**C**) embryo proper and cotyledons, and (**D**) endosperm in dehulled type II buckwheat seeds ($n = 5$ each). Peaks: 1, catechin; 2, epicatechin, IS ethyl gallate (IS); 3, epicatechin gallate; and 4, rutin.

presented just one oxidation wave, at +0.5 V. In the case of rutin, a current at a potential more positive than +0.5 V was constant. The potential value +0.5 V vs Ag/AgCl gave the best signal-to-noise ratio (S/N) for determining rutin, while the potential value +0.9 V vs Ag/AgCl gave the best S/N for catechin, epicatechin, and epicatechin gallate. For potentials more positive than +0.9 V vs Ag/AgCl, sensitivity was higher, but reproducibility was less, possibly due to the roughness of the electrode surface caused by oxidation of the glassy carbon working electrode surface. For highly sensitive determination without loss of selectivity and reproducibility, the potential value +0.5 V vs Ag/AgCl was adopted for the present study.

An examination was made of how the ratio of water, acetonitrile, and formic acid in the mobile phase influenced the separation for determination of rutin, catechin, epicatechin, and epicatechin gallate. The larger the content of water, the longer was the retention time of the rutin peak. To determine rutin, catechin, epicatechin, and epicatechin gallate in buckwheat flour and buckwheat seeds with adequate resolution within a short time, a mixture of acetonitrile−water−formic acid (13:87:1, v/v/v) was chosen as the most suitable mobile phase.

A typical chromatogram for a standard solution of rutin, catechin, epicatechin, epicatechin gallate, and ethyl gallate (IS) is shown in **Figure 3A**. The retention times of catechin, epicatechin, ethyl gallate (IS), epicatechin gallate, and rutin were 5.7, 8.8, 13.8, 23.5, and 29.4 min, respectively. Peak heights were found to be linear with respect to concentrations of each compound: rutin, 2 ng/mL to 2 μg/mL, correlation coefficient (r), $r = 0.999$, relative standard deviation (RSD) = 0.01−0.58%; catechin, 1 ng/mL to 1 μg/mL, $r = 0.999$, RSD = 0.01−1.52%; epicatechin, 1 ng/mL to 1 μg/mL, $r = 0.999$, RSD = 0.05−0.25%; ethyl gallate (IS), 1 ng/mL to 1 μg/mL, $r = 0.999$, RSD = 0.22−1.38%; and epicatechin gallate, 500 pg/mL to 500 ng/mL, RSD = 0−1.66%, $r = 0.999$. The detection limit (S/N = 3) of the present method for rutin was 0.86 ng/mL. The detection limit of rutin by the present method was more sensitive than HPLC-DAD (*4*, *5*), RF-MEKC-UV (*6*), CE-UV (*7*), CE-ECD (*8*), CZE-AD (*9*), and HPLC-ECD (*10*).

This method is highly sensitive because the microbore column avoids diffusing samples and slows the flow rate, as compared with the conventional column (internal diameter, 4.0−4.6 mm). Thus, highly concentrated and undiluted analyte reaches the surface of the working electrode, increasing the electrolytic efficiency of analyte on the working electrode.

Determination of Rutin, Catechin, Epicatechin, and Epicatechin Gallate in Buckwheat Flour. Rutin, catechin, epicatechin, and epicatechin gallate were determined by μHPLC-ECD method. A typical chromatogram for buckwheat flour is shown in **Figure 3B**. Rutin, catechin, epicatechin and epicatechin gallate contents in buckwheat flour are listed with their recovery data in **Table 1**. The RSDs for rutin, catechin, epicatechin, and epicatechin gallate peak height were less than 0.86% ($n = 5$). Rutin, catechin, epicatechin, and epicatechin gallate recoveries from spiked test solutions were more than 90%, and RSDs were less than 0.86% ($n = 5$). The results demonstrate that the μHPLC-ECD method is characterized by higher reproducibility, indicating that the present μHPLC-ECD method provides quite accurate measurements of rutin, catechin, epicatechin, and epicatechin gallate in buckwheat flour.

Distribution of Rutin, Catechin, Epicatechin, and Epicatechin Gallate in Buckwheat Seeds. Each part of a buckwheat seed contains different concentrations of rutin, catechin, epicatechin, and epicatechin gallate. In this study, we determined the concentrations of rutin, catechin, epicatechin, and epicatechin gallate from the bottom part of endosperm, testa, embryo proper and cotyledons, and endosperm. Also, to determine the concentrations of these compounds, we chose two types of dehulled buckwheat seeds: type I and type II.

The type I buckwheat seeds have a green testa, while type II buckwheat seeds have a brown testa. **Figures 4** and **5** show the

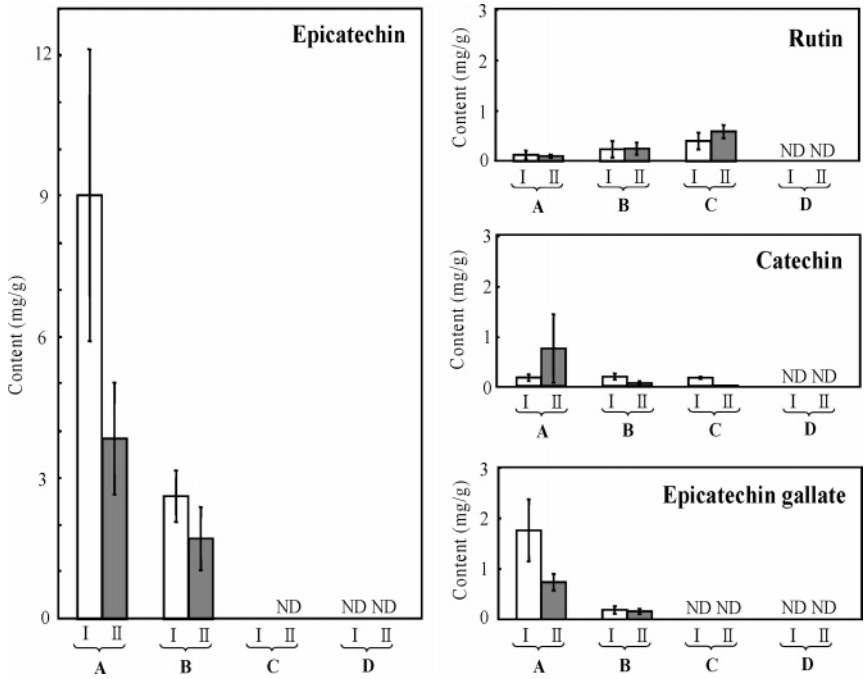

Figure 6. Mean (±SE) content of rutin, catechin, epicatechin, and epicatechin gallate in (**A**) the bottom part of the endosperm, (**B**) testa, (**C**) embryo proper and cotyledons, and (**D**) endosperm in dehulled types I and II buckwheat seeds ($n = 5$ each). ND indicates not detected.

chromatograms of rutin, catechin, epicatechin, and epicatechin gallate in (**A**) the bottom part of endosperm, (**B**) testa, (**C**) embryo proper and cotyledons, and (**D**) endosperm in dehulled types I and II buckwheat seeds ($n = 5$ each), respectively. The embryo proper and cotyledons of types I and II buckwheat seeds contained higher concentrations of rutin than the other parts of the seed, while the highest concentrations of epicatechin and epicatechin gallate were present in the embryo proper and cotyledons and the testa of the types I and II buckwheat seeds. The endosperm did not contain any detectable concentrations of these compounds. The re-extraction of extracted sample of buckwheat seeds except for testa showed that rutin, catechin, epicatechin, and epicatechin gallate were extracted almost 100% by the present procedure. In the case of testa, rutin, catechin, epicatechin, and epicatechin gallate were extracted by the present procedure by 91, 100, 99, and 100%, respectively. Therefore, the extraction efficiency of rutin, catechin, epicatechin, and epicatechin gallate from buckwheat seeds seems to be enough.

Figure 6 summarizes mean (± standard error, SE) contents of rutin, catechin, epicatechin, and epicatechin gallate in (**A**) the bottom part of endosperm, (**B**) testa, (**C**) embryo proper and cotyledons, and (**D**) endosperm dehulled types I and II buckwheat seeds ($n = 5$ each). ND indicates not detected.

In this study, the μHPLC-ECD method was established as a sensitive, selective, and accurate method for the determinations of rutin, catechin, epicatechin, and epicatechin gallate in buckwheat flour and buckwheat seeds. Also, the present method can be applied to the determination of rutin and epicatechin in buckwheat leaves, flowers, and roots. Moreover, the present method was applied to the distribution analysis of these compounds in a buckwheat seed. The embryo proper and cotyledons of a mature buckwheat seed contained rutin with the highest concentration as compared with the other parts. Environmental conditions such as soil and climatic requirements (*19*), and rooting depth and water use (*3*), can influence buckwheat crops. Using this method, the most suitable environmental conditions for buckwheat crops can be predicted as well as the best times to harvest can be predicted. Thus, using a small sample amount, this method is an important contribution to the cropping system and for quality control in the food and agricultural industries.

ABBREVIATIONS USED

CE, capillary electrophoresis; CZE, capillary zone electrophoresis; DAD, diode array detection; ECD, electrochemical detection; IS, internal standard; μHPLC, micro-high-performance liquid chromatography; RF-MEKC-UV, reverse-flow micellar electrokinetic chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; RSD, relative standard deviation; SE, standard error.

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