Prev. Nutr. Food Sci. 2019;24(1):75-83
https://doi.org/10.3746/pnf.2019.24.1.75
pISSN 2287-1098 · eISSN 2287-8602

Influence of Mineral Supplementation on the Results from Analysis of Flavonol Glycoside Content in *Ginkgo biloba* Dietary Supplements

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ABSTRACT: Flavonoids are a major component of *Ginkgo biloba* extract (GBE). Several studies have investigated chelate formation and the redox reaction between flavonoids and metal ions; however, the effect of mineral supplements on the results from the analysis of the flavonol glycoside content in products containing GBE dietary supplement remains unknown. In this study, the effects of commonly used mineral supplements on the recovery of quercetin from GBE-containing dietary supplements were investigated using conventional methods of flavonol glycoside determination. Mineral supplements containing Zn (II), Mn (II), and Fe (II) did not affect quercetin recovery, whereas Cu (II) and Fe (III) significantly reduced recovery ($P<0.05$). Quercetin oxidation was prevented by adding an antioxidant to the diluent (extraction solvent). Among the tested synthetic antioxidants, *tert*-butyl hydroquinone (TBHQ) promoted the greatest increase in quercetin recovery. The flavonol glycoside content of commercially available GBE-containing dietary supplements was analyzed using a conventional diluent or a diluent containing 20 mg/mL TBHQ. The amount of quercetin recovered from products containing Cu (II) was found to decrease with increasing hydrolysis duration and the duration in the final test solution state using the conventional diluent, while the TBHQ-containing diluent yielded consistent quercetin contents ($P<0.05$). These findings suggest that quercetin, a major aglycone of GBE flavonol glycosides, can be oxidized by Cu (II) and Fe (III) during the analytical process and, therefore, the total flavonol glycoside content may be underestimated. The addition of TBHQ to the diluent can improve the accuracy and reproducibility of flavonol glycoside content analysis in GBE-containing dietary products supplemented with minerals.

Keywords: *Ginkgo biloba*, flavonol glycoside, quercetin, oxidation, TBHQ

INTRODUCTION

Ginkgo biloba is one of the oldest living tree species and is considered a "living fossil"; its natural habitat is in China, Japan, and Korea. In the 18th century, it was introduced into Europe and the United States, and has been cultivated as an ornamental tree and a medicinal plant (1,2). The leaves and seeds of *Ginkgo biloba* have been used in traditional Chinese medicine in the treatment of asthma, bronchitis, and fatigue for several years. Moreover, extracts of the leaves are used in treatment for cardiovascular diseases, allergies, tinnitus, peripheral arterial occlusive diseases, cerebral insufficiency, and dementias such as Alzheimer's disease (3-12).

Ginkgo biloba extract (GBE) is used in dietary supplements in addition to medicine. In Korea, GBE can be used as a constituent of health/functional food (HFF), which is regulated by the HFF Act of Korea. The term HFF refers to food supplements containing nutrients or other substances in a concentrated form that have nutritional or physiological effects, the purpose of which are to supplement normal diet. The Ministry of Food and Drug Safety (MFDS) of Korea has approved claims that GBE-containing HFFs improve memory and blood circulation (13,14).

Two major bioactive compounds in GBE are flavonoids and terpene lactones; the flavonoids are present primarily as glycosides. Therefore, quality control of GBE has been standardized for these two components. GBE contains more than 20 flavonoid glycosides, most of which

Received 6 November 2018; Accepted 7 January 2019; Published online 31 March 2019

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are derived from quercetin, kaempferol, and isorhamnetin (2,3,15). In several standardized protocols to evaluate the quality of *Ginkgo* leaf and its related products, the total flavonol glycoside content was calculated from the content of the three primary aglycones: quercetin, kaempferol, and isorhamnetin (14-18).

Several HFF products containing GBE manufactured and sold in Korea also contain minerals such as zinc, iron, and copper (19). Furthermore, flavonoids are known to form chelate complexes with metal ions, and these chelates are known to result in increased bioavailability of flavonols and metals (20). In addition, it has been reported that iron and copper promote quercetin oxidation under low pH and increasing temperatures (21-23). Therefore, the aglycones of flavonol glycosides can be determined after acid hydrolysis and heating (14,15,17,18, 24), which represents an oxidizing condition for both metal ions and flavonol aglycones. If GBE products also contain mineral supplements such as iron and copper, quercetin oxidation may occur after hydrolysis, affecting the quantitative analysis of flavonol glycosides. However, the effect of mineral supplements on the results from the analysis of flavonol glycoside content in GBE-containing dietary supplement products is not yet known.

In the present study, the effect of mineral supplements on the analysis of flavonol glycosides and methods to suppress this effect were investigated.

MATERIALS AND METHODS

Materials

Absolute ethanol and methanol were purchased from Fisher Scientific Korea (Seoul, Korea). Hydrochloric acid (HCl; 35%) was purchased from Matsunoen Chemicals Ltd. (Osaka, Japan). Phosphoric acid (85%), iron (II) fumarate, and manganese (II) sulfate pentahydrate were purchased from Wako Pure Chemical Corporation (Tokyo, Japan). Iron (III) chloride hexahydrate, iron (II) sulfate heptahydrate, butylated hydroxytoluene (BHT) and ethylenediaminetetraacetic acid (EDTA) were purchased from Junsei Chemical (Tokyo, Japan). Copper (II) sulfate and zinc (II) oxide were purchased from Kanto Chemical (Tokyo, Japan). Zinc (II) gluconate hydrate and tert-butyl hydroquinone (TBHQ) were purchased from Alfa Aesar (Tewksbury, MA, USA). Butylated hydroxyanisole (BHA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich USA (St. Louis, MO, USA). Copper (II) D-gluconate was purchased from Sigma-Aldrich Productions GmbH (Steinheim, Germany). Propyl gallate (PG) was purchased from Sigma-Aldrich China (Shanghai, China). Quercetin hydrate, the reference material for recovery test of quercetin, was purchased from Sigma-Aldrich USA. Quercetin dihydrate, kaempferol and isorhamnetin analytical standards to determine the flavonol aglycones content were purchased from HWI Pharma Service (Ruelzheim, Germany), Sigma-Aldrich USA and Sigma-Aldrich USA, respectively. Methanol of gradient grade for LC was purchased from Merck (Darmstadt, Germany). GBE dietary supplement product samples were purchased from online stores (Table 1).

Preparation of reagents

Diluent (extraction solvent): The diluent used was ethanol : water : hydrochloric acid (50:20:8, v/v/v) (14,16,17). For the preparation of approximately 780 mL diluent, 500 mL ethanol, and 200 mL deionized water were added into a 1 L bottle, before 80 mL HCl (35%) was added carefully and mixed thoroughly.

Mineral supplement solutions: Iron (III) chloride hexahydrate, iron (II) fumarate, iron (II) sulfate heptahydrate, copper (II) D-gluconate, copper (II) sulfate, manganese (II) sulfate pentahydrate, zinc (II) oxide, and zinc (II) gluconate hydrate were individually dissolved in the diluent to produce anhydrous solutions containing 10 mg/mL of each mineral supplement. These solutions were further diluted with the diluent as necessary.

Antioxidant solutions: The antioxidants TBHQ, BHT, BHA, PG, and EDTA were individually dissolved in the diluent to yield solutions of approximately 10 mg/mL each. Because EDTA and BHA were insoluble in the diluent, these were dissolved in a solution of water : hydrochloric acid (20:8, v/v) first, before ethanol was added to restore the composition of the diluent.

Diluent (extraction solvent) containing 20 mg/mL (w/v) TBHQ: This diluent was prepared by dissolving 15.6 g TBHQ in 780 mL diluent and mixing thoroughly.

Quercetin hydrate solution (reference material for quercetin recovery testing): To prepare this solution, 0.6 g quercetin hydrate was dissolved in 3 mL DMSO with sonication for complete dissolution, diluted using 50 mL diluent, and mixed thoroughly. This solution contained approximately 10 mg/mL quercetin.

Quercetin dihydrate, kaempferol, and isorhamnetin standard stock solutions (analytical standards to determine flavonol aglycone content): Quercetin dihydrate, kaempferol, and isorhamnetin analytical standards were dissolved in 2 mL DMSO and sonicated for 2 min to dissolve the solids. Solutions were diluted to 100 mL with methanol and mixed thoroughly. The amount of quercetin dihydrate converted to quercetin was calculated as [quercetin (mg)=quercetin dihydrate (mg)×302/338]. The stock solutions were diluted with methanol to construct a five-point calibration curve with concentrations ranging from 4.438 to 110.96 μg/mL for quercetin, 4.656 to 116.4 μg/mL for kaempferol, and 0.792 to 19.8 μg/mL for isorhamnetin. The standard solutions were stored at 4°C for up to 6 months, and equilibrized at room temperature before use.

Table 1. *Ginkgo biloba* dietary supplement products used in this study

Sample No.	Label claim				Others	Type
	Serving size	Flavonol glycosides	Mineral supplements			
			Element	Raw material		
2017S_FG_1	310 mg	14.4 mg	–	–	–	Tablet
2017S_FG_2	1,000 mg	28 mg	Zinc 8.5 mg Iron 6 mg Manganese 3.5 mg Copper 0.5 mg	Zinc oxide Ferrous fumarate Manganese sulfate Copper sulfate	Selenium 55 μg	Tablet
2017S_FG_3	1,000 mg	28 mg	Zinc 10 mg Iron 4.5 mg	Zinc oxide Ferrous fumarate	Vitamin C 45 mg	Tablet
2017S_FG_4	500 mg	28.8 mg	Zinc 4.25 mg	Zinc oxide	Vitamin E 5.5 mg α-TE	Capsule
2017S_FG_5	700 mg	30 mg	–	–	Selenium 70 μg Vitamin C 30 mg Vitamin D 50 μg Vitamin B6 1.6 mg Vitamin B12 10 μg	Tablet
2017S_FG_6	600 mg	36 mg	Zinc 3.6 mg Iron 4.5 mg Manganese 0.9 mg Copper 0.24 mg	Zinc oxide Ferrous fumarate Manganese sulfate Copper sulfate	Selenium 16.7 μg Vitamin B1 0.36 mg Vitamin B2 1.4 mg Niacin 15 mg NE Pantothenic acid 5 mg Vitamin B6 1.5 mg	Tablet
2017S_FG_7	500 mg	36 mg	Zinc 3.57 mg Iron 4.56 mg Manganese 1.29 mg Copper 0.344 mg	Zinc oxide Ferrous fumarate Manganese sulfate Copper sulfate	Selenium 19.8 μg Vitamin B1 1.2 mg Vitamin B2 1.4 mg Niacin 15 mg NE Pantothenic acid 5 mg Vitamin B6 1.5 mg	Tablet

α-TE, alpha-tocopherol equivalent; NE, niacin equivalent.

All solutions used in this study were prepared freshly before use except for the standard solutions.

Preparation of analytical samples

Influence of mineral supplements on quercetin recovery: Samples for evaluation of the influence of each mineral supplement on quercetin recovery were prepared as follows. Solutions of 8 mL diluent and 1 mL of 10 mg/mL quercetin hydrate were added into 10 mL conical tubes, and 1 mL of each 10 mg/mL mineral supplement solution was added into each individual tubs.

Samples for evaluation of the influence of iron and copper supplement concentrations on quercetin recovery were prepared as follows. Solutions of 8 mL diluent and 1 mL of 10 mg/mL quercetin hydrate were added into 10 mL conical tubes, before iron (III) chloride, iron (II) fumarate, or copper (II) sulfate solution was added to final concentrations of 0.05, 0.25, 0.5, and 1 mg/mL. Further diluent was added to a total volume of 10 mL.

As a control, 1 mg/mL quercetin hydrate containing diluent was prepared. Assuming the quercetin recovery from the control was 100%, the relative recovery of each sample containing the mineral supplements were determined.

Suppression of quercetin oxidation due to addition of antioxidants in the diluent: Samples for evaluation of the suppressive effects of each artificial antioxidant on quercetin oxidation were prepared as follows. Solutions of 8 mL diluent and 1 mL of 10 mg/mL quercetin hydrate were added into 10 mL conical tubes, and EDTA, TBHQ, BHA, BHT, or PG solution were added to final concentrations of 2 and 4 mg/mL. Iron (III) chloride was added to a concentration of 0.5 mg/mL, and was topped up with further diluent to a total volume to 10 mL.

Samples for evaluating the suppressive effect of TBHQ concentration on quercetin oxidation were prepared as follows. Solutions of 4 mL diluent and 1 mL of 1 mg/mL quercetin hydrate were added into 10 mL conical tubes. 50 mg/mL TBHQ solution was added to final concentrations of 5, 10, 15, and 20 mg/mL. Solutions of 1 mL of 10 mg/mL iron (III) chloride, 10 mg/mL iron (II) fumarate, 10 mg/mL copper (II) sulfate, or 1 mg/mL copper (II) sulfate were then added, and topped up with further diluent to a total volume of 10 mL.

As a control, 1 mg/mL quercetin hydrate and 0.5 mg/mL iron (III) chloride containing diluent was prepared. Assuming the quercetin recovery from 1 mg/mL quercetin hydrate containing the diluent used in above was 100%, we were able to determine the relative recoveries of the samples and control.

Each prepared sample was placed in a water bath at 90 °C for 60 min for hydrolysis, and then cooled to an ambient temperature. A portion of the hydrolyzed solution was diluted 10-fold with methanol, filtered through 0.45

μm polyvinylidene difluoride (PVDF), and analyzed using a high-performance liquid chromatography (HPLC).

Comparison of total flavonol glycoside content in GBE-containing dietary supplement products hydrolyzed with TBHQ-containing diluent or conventional diluent: Test solutions of seven GBE-containing supplement products were prepared using the MFDS method. Tablet or capsule contents were ground and passed through a no. 60 sieve, before 0.3 g (±0.02 mg) of the ground samples were transferred into flasks. A solution of 10 mL conventional diluent or 20 mg/mL TBHQ-containing diluent was added and mixed thoroughly. The contents were hydrolyzed by placing the flasks in a water bath at 90°C for 60 min, and then removed and cooled to an ambient temperature. The contents were transferred into a 50 mL volumetric flask, and the total volume made up to 50 mL with methanol and mixed well. A portion of the solution was filtered using a 0.45 μm PVDF into an autosampler vial.

Change in total flavonol glycoside content determined in GBE-containing dietary supplement products based on hydrolysis duration and diluent: Sample no. 2017S_FG_2 was used with a hydrolysis duration of 30, 45, 60, 75, 90, or 120 min. The rest of the analytical procedure were as described in above.

HPLC conditions

An Agilent 1200 series HPLC system and the Agilent OpenLab CDS V2.1 software (Agilent Technologies, Santa Clara, CA, USA) were used. The HPLC conditions were as follows: column temperature, 35°C; flow rate, 1.0 mL/min; injection volume, 10 μL; detection wavelength, 370 nm for detection of flavonol aglycones and 291 nm for oxidative compounds; run time, 30 min; HPLC column, UG120 (Capcell Pak C18, 5 μm, 4.6×250 mm; Shiseido, Tokyo, Japan); mobile phase (isocratic) methanol : water : phosphoric acid (85%)=100:99:1 (v/v/v).

Determination of total flavonol glycoside content in GBE-containing supplement products

The total flavonol glycoside concentration was determined from the combined peak areas of quercetin, kaempferol, and isorhamnetin at 370 nm. Aglycone conversion factors (2.504 for quercetin, 2.588 for kaempferol, and 2.437 for isorhamnetin) were adopted from previous standardized protocols (14,17).

Statistical analysis

All experimental values shown are the means of three independent replicates, and the error bars are standard deviations. Statistically significant differences between experimental mean values were analyzed using IBM SPSS Statistics 24 software (IBM Corp., Armonk, NY, USA) using one-way ANOVA, and multiple comparisons were calculated using Tukey's post hoc test. The threshold for statistical significance was $P<0.05$.

RESULTS AND DISCUSSION

Influence of mineral supplements on quercetin recovery

The recoveries of quercetin from the mineral-supplemented samples, relative to recovery from the control, were as follows: copper (II) sulfate, 0%; copper (II) D-gluconate, 39.5%; iron (III) chloride, 2.5%, iron (II) fumarate, 75.4%; and iron (II) sulfate, 81.2%. The recoveries of samples supplemented with zinc (II) oxide, zinc

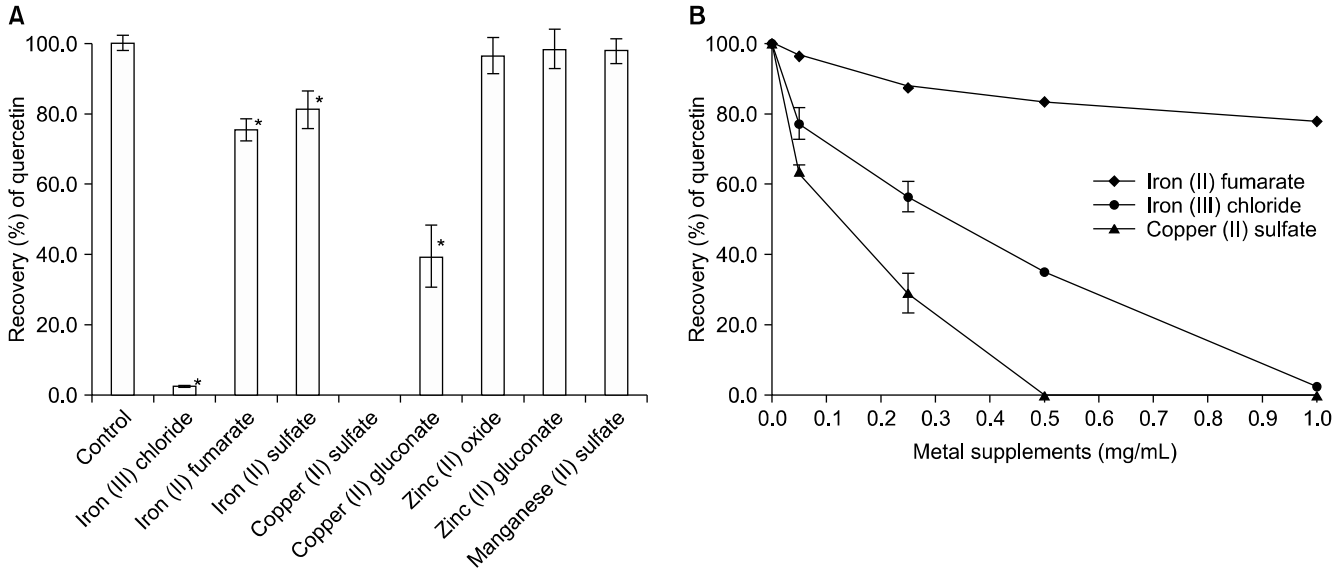

Fig. 1. Reduced recovery of quercetin by (A) mineral supplements and (B) concentration of iron (II) fumarate, iron (III) chloride, and copper (II) sulfate. The described concentration is the concentration in the diluent. All values shown are the mean of three independent replicates, and the error bars are the mean±standard deviation. *Significantly lower than control at $P<0.05$.

(II) gluconate, or manganese (II) sulfate were not significantly different from the control. Whilst zinc (II) and manganese (II) had no effect on recovery, copper (II) and iron (III) reduced recovery significantly ($P<0.05$), presumably due to quercetin oxidation (Fig. 1A).

The effects of iron (III) chloride, iron (II) fumarate, and copper (II) sulfate on quercetin recovery were next investigated by varying the concentrations of these supplements from 0.05 to 1 mg/mL; the greater the amount of supplemented mineral, the greater the reduction in quercetin recovery (Fig. 1B).

Balcerzak et al. (21) developed a selective ultraviolet (UV)-spectrophotometric method for determination of iron (III) in iron (II)-containing pharmaceutical products. This method is based on the redox reactions occurring between iron (III) ions and quercetin. The authors reported that iron (II) did not react with quercetin under the experimental conditions used [aqueous : methanolic (3:2) solutions, 0.3 M HCl]. Moreover, that the equilibrium in both complexation and the redox reactions between iron (III) and quercetin depend on the hydrochloric acid concentration, reaction time, and temperature. Lower acid concentrations were favorable for formation of the iron (III)-quercetin complex. An increase in HCl concentration in solutions containing the complex shifts the equilibrium towards the redox process, leading to quantitative formation of the final oxidized form of quercetin. The optimum HCl concentrations for formation of the complex and product in the redox reaction are 0.03 M and 0.3 M, respectively. Furthermore, redox reactions can be significantly accelerated by heating. The HCl concentration in the diluent used in this study was 1.2 M, which decreased to 0.24 M when diluted 5-fold with methanol per the flavonol glycoside determination method of the MFDS. Compared with those previously reported (21), the concentrations in the present study represent conditions under which redox reactions are more favorable than chelation. Liu and Guo (23) reported that the stability of quercetin-divalent metal ion complexes decreased in the following order: Cu (II)>Ni (II)>Co (II)>Fe (II)>Zn (II). At a flavonoid : metal stoichiometry ratio of 2:1, complexes formed between quercetin and metal ions [Fe (II), Ni (II), Co (II), and Zn (II)] had similar fragmentation mechanisms; whereas Cu (II) showed a different fragmentation mechanism due to concurrent oxidation, which was partially because Cu (II) has stronger oxidative activity and quercetin is easier to oxidize under acidic conditions. Fernandez et al. (25) suggested that the higher yield generated in redox reactions with copper rather than iron is consistent with the lower redox potential of Cu (II)/Cu (I) compared with that of Fe (III)/Fe (II).

The reduced recovery of quercetin in the presence of iron (II) fumarate and iron (II) sulfate divalent irons, is discussed in the subsequent results.

Suppression of quercetin oxidation due to addition of antioxidants to the diluent

Among the tested antioxidants, quercetin recovery was highest when TBHQ was added to the diluent containing iron (III) chloride (Fig. 2A), whereby the higher the TBHQ concentration, the higher the quercetin recovery (Fig. 2B). While the quercetin recovery of the diluent without TBHQ was 0% (not detected), TBHQ-containing diluents exhibited recoveries of 92~102% of the control.

Hertog et al. (26), Merken and Beecher (27), Toku et al. (28), and Kwak et al. (29) also used TBHQ in studies of vegetable and fruit flavonoids. The concentrations used in these studies ranged from 0.4 to 2 mg/mL, which are much lower than those used in the present study. Our results show that a larger amount of TBHQ is required to inhibit oxidation reactions in samples containing high

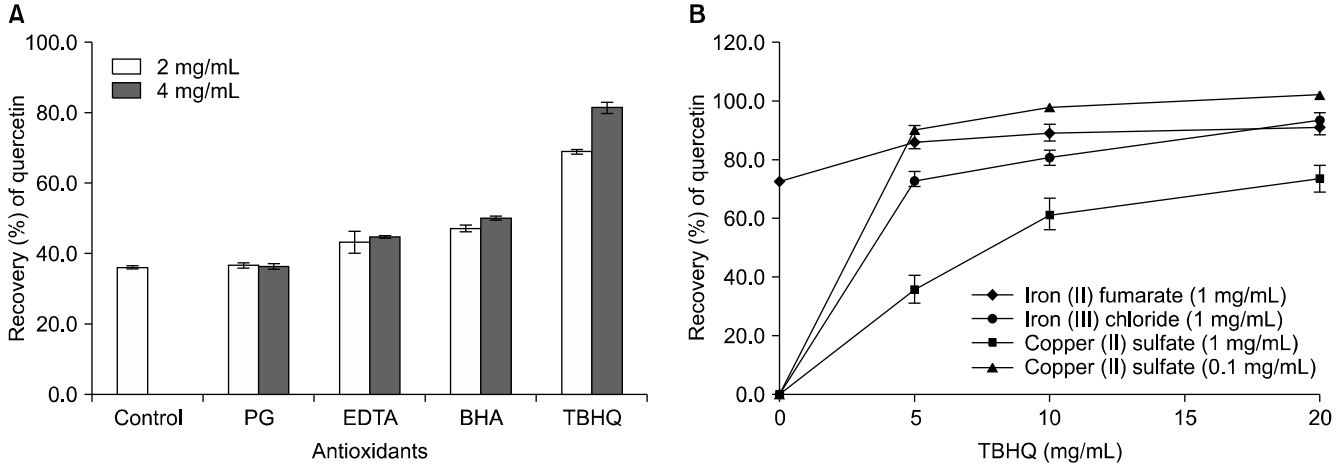

Fig. 2. Inhenced recovery of quercetin by (A) antioxidants and (B) concentration of *tert*-butyl hydroquinone (TBHQ). The described concentration is the concentration in the diluent. PG, propyl gallate; EDTA, ethylenediaminetetraacetic acid; BHA, butylated hydroxyanisole. All values shown are the mean of three independent replicates, and the error bars are the mean±standard deviation.

concentrations of metals, such as HFF samples.

The concentrations of quercetin and mineral supplements used in quercetin recovery testing were determined with reference to their concentrations in GBE-containing HFFs manufactured and sold in Korea. The contents per serving size in 108 GBE products registered with the MFDS are 28～36 mg flavonol glycosides, 3.63～6.70 mg iron, and 0.24～0.34 mg copper, if added. When these were analyzed using the MFDS testing method, the concentrations in the conventional diluent were 0.03～0.96 mg/mL quercetin (corrected using the conversion factor for flavonol glycoside to quercetin, assuming that only the quercetin aglycone was present), approximately 0.35～0.85 mg/mL iron (III) chloride or iron (II) fumarate, and 0.03～0.05 mg/mL copper (II) sulfate.

Comparison of TBHQ-containing and conventional diluents

The flavonol glycoside content in seven GBE-containing supplement products was measured over time. The flavonol glycoside content of the products containing no mineral supplements (2017S_FG_1, 2017S_FG_5), and of those containing zinc (II) supplement (2017S_FG_4), or zinc (II) and iron (II) supplements (2017S_FG_3), did not change over time. However, three products supplemented with copper (2017S_FG_2, 2017S_FG_6, and 2017S_FG_7) showed a decrease in total flavonol glycoside content over time ($P<0.05$) (Table 1 and Fig. 3).

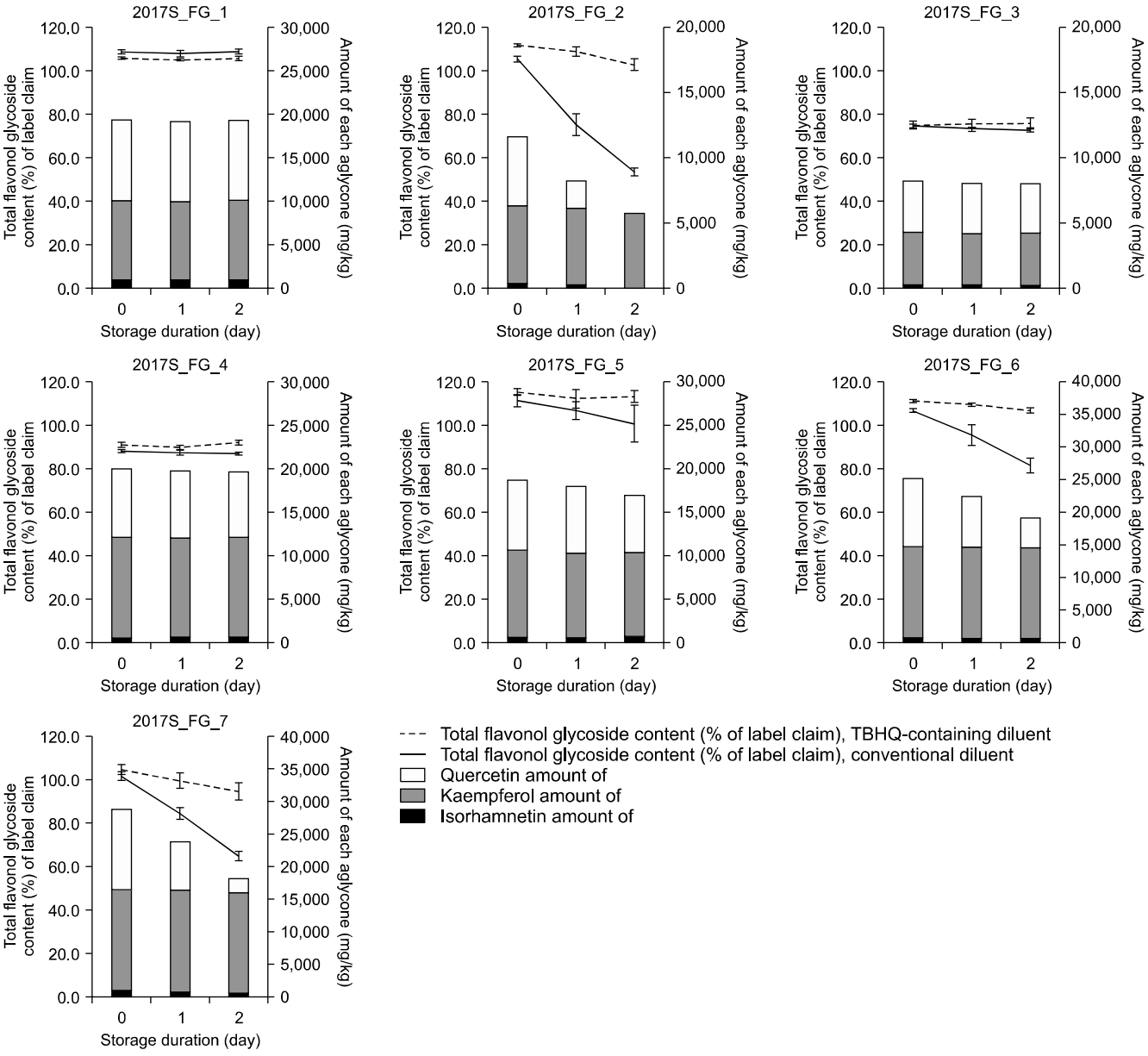

Fig. 3. Change of total flavonol glycoside contents in the final test solution of *Ginkgo biloba* dietary supplement products during storage periods hydrolyzed by *tert*-butyl hydroquinone (TBHQ) added diluent and conventional diluent. Bar graphs indicate the amount of each flavonol aglycone which was hydrolyzed by conventional diluent. All values shown are the mean of three independent replicates, and the error bars are the mean±standard deviation.

The reduction in flavonol glycoside content varied depending on the product. Relative to the claim on the label, the total content in 2017S_FG_2 decreased from 105.8% (immediately after preparing the test solution) to 75.5% after 1 day, and to 53.7% after day 2. This decrease was due to a decrease in the amount of quercetin; the effect of kaempferol or isorhamnetin was negligible. However, when 20 mg/mL of TBHQ was added to the diluent, the amount of quercetin remained relatively constant: 112.0% immediately after preparation, 109.1% after 1 day, and 103.1% after day 2. The other two samples containing copper, 2017S_FG_6 and 2017S_FG_7, showed similar results to 2017S_FG_2.

We did not detect any changes in the amount of kaempferol. The amount of quercetin in the solution decreased due to rapid oxidization under test conditions, whereas kaempferol was stable. Fernandez et al. (25) reported that quercetin has a greater reducing ability than kaempferol.

In previous studies, quercetin recovery was reduced due to iron (II) fumarate and iron (II) sulfate. However, the results of our analysis of 2017S_FG_3, a product containing iron (II) fumarate, showed no changes in the amount of quercetin over time, even without TBHQ addition. It therefore appears that iron (II) fumarate is not directly involved in quercetin oxidation. An explanation for the decrease in quercetin recovery in previous studies using iron (II) fumarate may be due to oxidization of iron (II) to iron (III) over the course of the experiment, or the presence of a trivalent iron in the reagent. According to the manufacturer's certificate of analysis, the reagent contains 0.5 % iron (III).

Jungbluth et al. (30) reported that quercetin may be oxidized in the presence of Cu (II) or Fe (III) in aqueous media, but Fe (II) causes no immediate change in the UV/Vis spectra of the flavonoids. The authors suggested that the presence of iron in either oxidation state may lead to transformation of flavonols due to possible oxidation of Fe (II) to Fe (III) through molecular oxygen.

Change in total flavonol glycoside content in GBE-containing dietary supplement products based on hydrolysis duration and diluent

The total flavonol glycoside content in 2017S_FG_2 was measured after different durations of hydrolysis. When the conventional diluent was used, the amount of quercetin began to decrease at 60 min, and showed a significant difference from 75 min ($P<0.05$) (Fig. 4). Therefore, in the analysis of copper-supplemented products, the flavonol glycoside content may be underestimated due to prolonged hydrolysis. However, when TBHQ was added to the diluent, the flavonol glycoside content was consistent regardless of the duration of hydrolysis.

Hertog et al. (26) optimized a quantitative HPLC-based method for determination of flavonoid aglycones in vegetables and fruits following acid hydrolysis of glycosides. The authors reported that increasing the acid concentration and reaction time of the acid hydrolysis led to significant quercetin degradation in sample solutions. However, flavonol degradation was not observed in the standard solutions treated under identical conditions. Therefore, whether the presence of some unknown compounds in the sample matrix may have accelerated degradation of flavonoids should be considered. Vegetables and fruits naturally contain trace amounts of iron and copper; these metals could be the compounds accelerating degradation of flavonoids.

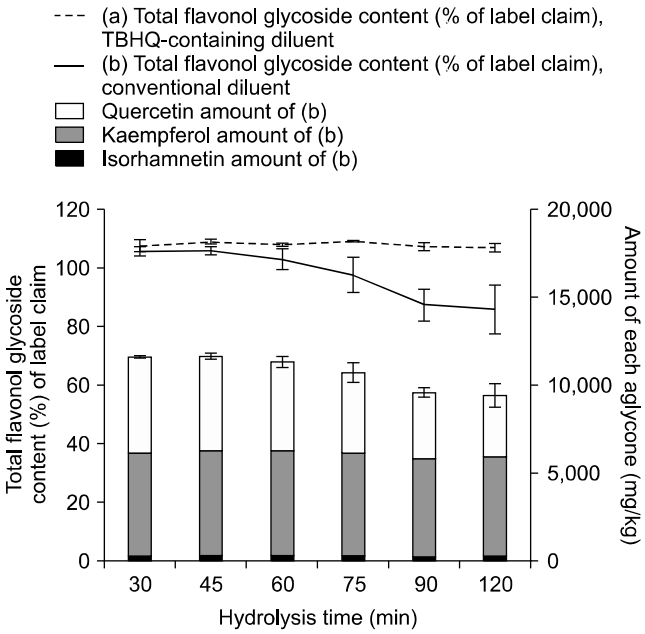

Fig. 4. Change of total flavonol glycoside contents of *Ginkgo biloba* dietary supplement products by hydrolysis time. Bar graphs indicate the amount of each flavonol aglycone which was hydrolyzed by conventional diluent. All values shown are the mean of three independent replicates, and the error bars are the mean±standard deviation. TBHQ, *tert*-butyl hydroquinone.

GBE-containing products supplemented with iron (III) were not available. However, iron (III) supplements, such as iron (III) chloride, have also been approved for iron fortification. In products containing iron (III), quercetin oxidation can occur during analysis, similar to in copper-containing products.

Fig. 5 shows chromatograms of sample No. 2017S_FG_7, obtained 2 days after preparation of test solutions. The peak elution at 5.13 min is presumed to be a product of quercetin oxidation, considering that the peak intensity was inversely proportional to the decrease in quercetin, and that the UV spectrum was similar to the spectrum of the oxidation products of quercetin (21,22). TBHQ eluted at approximately 9.53 min and was not detected at the aglycone assay wavelength of 370 nm, but was detected instead at 291 nm. TBHQ was also completely separated from quercetin (Rs=8), the closest peak, and,

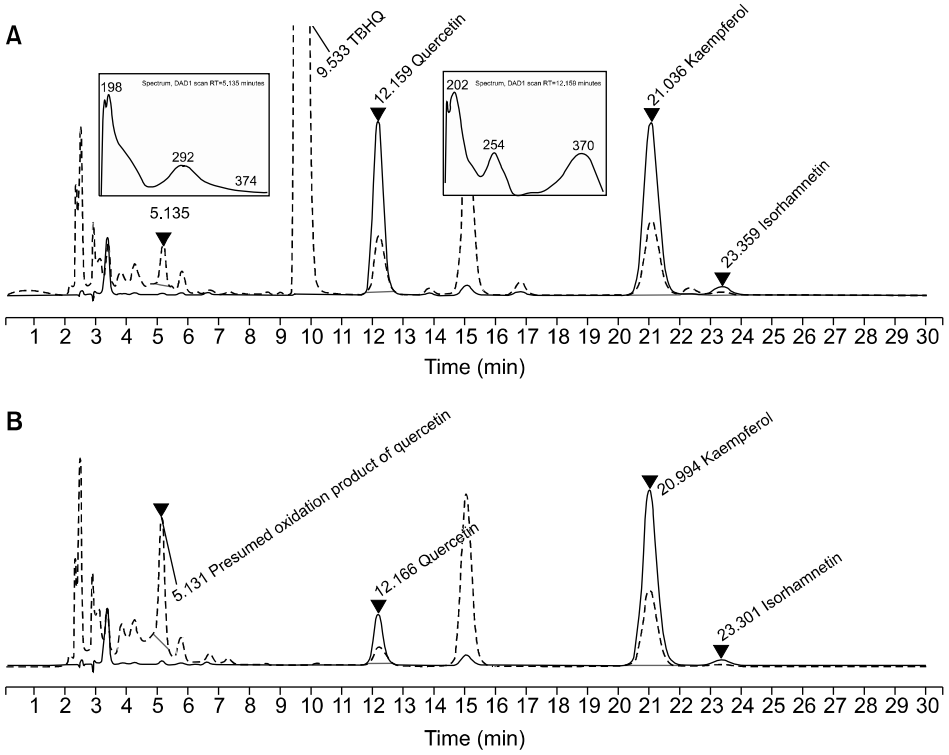

Fig. 5. Chromatogram of *Ginkgo biloba* dietary supplement product hydrolyzed with (A) *tert*-butyl hydroquinone (TBHQ) added diluent and (B) conventional diluent (solid line, 370 nm; dash line, 291 nm).

therefore, did not affect flavonol glycoside analysis.

The effects of eight mineral supplements commonly used in dietary supplement foods on the recovery of quercetin were investigated. Zn (II), Mn (II), and Fe (II) supplements did not affect quercetin recovery, but Cu (II) and Fe (III) supplements significantly reduced the recovery rate in a dose-dependent manner ($P<0.05$). This result may be due to copper and iron ions reacting with quercetin to accelerate its oxidation during analysis. As the quercetin is oxidized and degraded, the total flavonol glycoside content of GBE dietary supplement products may be measured as less than the amount actually present or that labelled on the product. As a result, an adequate product can be misjudged as a violated product with inadequate flavonol glycoside content.

Quercetin oxidation could be inhibited by adding an antioxidant during recovery. Of a total of four synthetic antioxidants, TBHQ showed the greatest increase in quercetin recovery, acting in a dose-dependent manner. The flavonol glycoside contents of seven commercially available GBE-containing dietary supplement foods were analyzed using a conventional diluent and a diluent containing 20 mg/mL TBHQ. The amount of quercetin recovered from three products supplemented with copper decreased with an increase in hydrolysis duration, and the duration of the final test solution state using the conventional diluent; whereas the TBHQ-containing diluent resulted in a consistent quercetin yield ($P<0.05$). A simple modification of the conventional flavonol glycoside determination method, such as addition of TBHQ to the diluent, can improve the accuracy and reproducibility of flavonol glycoside content analysis in GBE-containing dietary products supplemented with metals.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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