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Stability Evaluation of Selected Polyphenols and Triterpene Glycosides in Black Cohosh

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Black cohosh (*Actaea racemosa* L., syn. *Cimicifuga racemosa* L.) is rich in both triterpene glycosides and polyphenols, which have various biological activities that may be important to its medical use. To evaluate the stability of the polyphenolic constituents and triterpene glycosides of black cohosh, experiments were conducted using three sample types: plant material, extracts of black cohosh, and encapsulated commercial extract. The samples were stored at various temperatures and humidity conditions. Three triterpene glycosides and six major polyphenols in black cohosh were quantitatively measured with an HPLC-PDA method at 0, 3, 6, and 9 weeks. The triterpene glycosides were stable at the tested conditions, whereas the polyphenols were stable only at room temperature and low humidity and not stable at higher temperature and/or humidity due to hydrolysis and/or oxidation. The rate of compound decomposition depended upon the chemical structure of the individual polyphenols. Polyphenols in the extracts decomposed more readily than those in plant material.

KEYWORDS: Black cohosh; triterpene glycosides; polyphenols; stability; *Actaea racemosa*; *Cimicifuga racemosa*

INTRODUCTION

Black cohosh, *Actaea racemosa* L. (syn. *Cimicifuga racemosa* L.), a plant native to eastern North America, has a long history of medicinal use. This plant is presently used as an herbal medicine in North America and Europe for the treatment of menopausal symptoms, with a clinical use spanning the past 50 years (*1, 2*). Because of the risk of developing breast cancer and cardiovascular diseases for women who use long-term estrogen replacement therapy (*3, 4*), women turned to natural products, and black cohosh products have become increasingly popular as dietary supplements in the United States and Europe during the past several years (*5, 6*); retail sales of black cohosh herbal products in 2004 ranked eighth in terms of the U.S. botanical product market (*6*).

Although black cohosh now enjoys considerable popularity for the treatment of menopausal hot flashes, the mechanism of action of this plant for hot flashes is still not known. Many studies have examined the biological activity and bioactive constituents of black cohosh since the 1950s. Early studies focused mainly on the triterpene glycosides and examination of estrogenic activity. None of them demonstrated estrogenic activity. In the past two decades, more and better designed clinical studies have been conducted, but most studies are of short duration and relatively small, and the results have been mixed with respect to outcome (*7*−*13*). A clinical trial in China reported that an isopropanolic extract of black cohosh was as good as the synthetic steroid tibolone for the treatment of climacteric complaints (*14*). An in vitro study found that a triterpene glycoside from black cohosh showed beneficial effects on bone cells and could be a potential lead for the development of a new class of antiosteoporosis agents (*15*).

Some researchers are now focusing their attention on the polyphenolic constituents of black cohosh, and additional polyphenols continue to be isolated, including new phenylpropanoids and a lignan (*16*−*18*). Methods of extracting and detecting the polyphenolic constituents in black cohosh have also been reported (*19*−*21*). Black cohosh polyphenols possess a variety of biological activities. These compounds demonstrated antioxidant and anti-inflammatory activities (*18, 19, 22, 23*); black cohosh was determined to be one of the plants that showed the strongest antioxidant activity among common herbal medicines used to treat the urinary system (*24*). Black cohosh was historically used as a remedy for kidney problems, diarrhea, sore throat, and rheumatism, and the polyphenols are proposed to play an important role in these medical conditions (*24*−*26*). Other studies demonstrated that black cohosh polyphenols could

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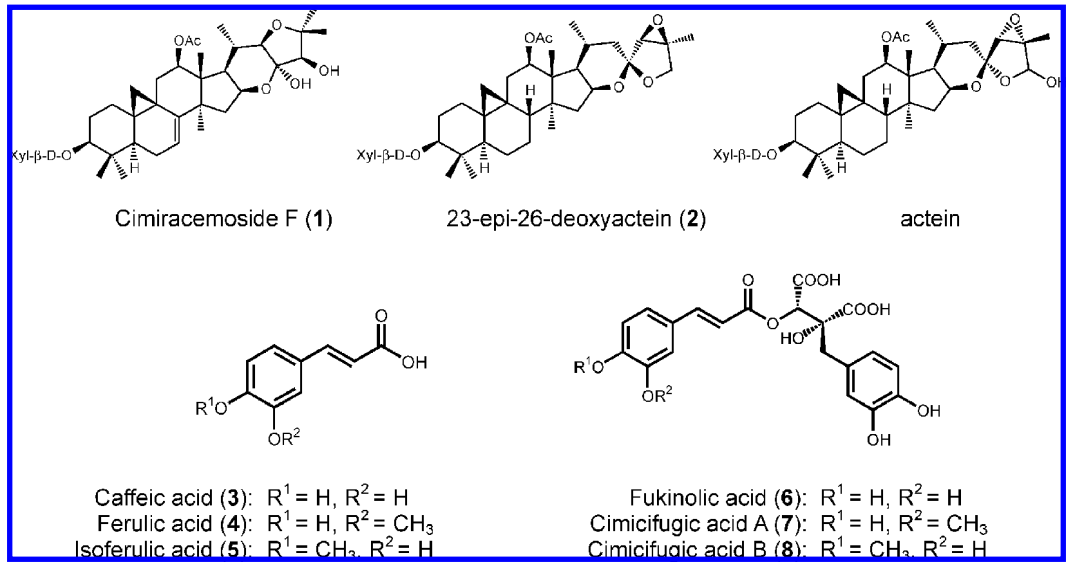

Figure 1. Three triterpene glycosides and six major phenolic constituents in black cohosh.

affect the enzymatic activities of α-amylase (*27*), collagenase (*28*), and neutrophil elastase (*29*) and inhibit the growth of LNCaP human prostate carcinoma cells (*17*).

In our previous research on the stability of black cohosh constituents, we found that triterpene glycosides were stable but that the amount of polyphenols in black cohosh samples changed over time (unpublished data). In an investigation of an 85-year-old black cohosh sample, we found that the total amount of six polyphenols was significantly lower than in a fresh sample but that the triterpenoid content was similar to that of a fresh black cohosh sample (*30*). Considering that any change in these constituents could potentially result in a change in some biological activity of black cohosh, it is, therefore, important to examine the stability of black cohosh constituents and assess the biological activities of black cohosh and the roles various constituents may play in clinical effectiveness and safe use.

MATERIALS AND METHODS

HPLC. The samples and standards were analyzed using a Waters 2695 HPLC (Milford, MA) equipped with a 996 photodiode array detector (PDA) and operated with Empower software. Separations were carried out on a 125 × 4.0 mm i.d. Hypersil ODS column (Agilent, Santa Clara, CA) for the quantitative analysis and on a 250 × 4.6 mm i.d., 5 μm Aqua C18 column (Phenomenex, Torrance, CA) for the qualitative analysis. Both analyses were performed at 25 °C with a flow rate of 1.0 mL/min. The sample volume injected was 10 μL, and data were analyzed at 203 nm for triterpene glycosides and at 280 or 320 nm for polyphenols. The mobile phase for the analysis of triterpene glycosides consisted of a step gradient starting with 5% (v/v) acetonitrile (solvent A) in water (B) and increasing to 100% acetonitrile over 60 min. The gradient profile was as follows: 0−40 min, 5−50% A; 40−45 min, 50−60% A; 45−55 min, 60−80% A; 55−60 min, 80−100% A. The solvent system for the quantitative analysis of polyphenols was composed of acetonitrile (A) and 10% aqueous formic acid (B) using a step gradient elution of 5−25% A at 0−24 min, 25−50% A at 24−25 min, and 50−100% A at 25−26 min; the step gradient for the qualitative analysis was 5−15% A at 0−15 min, 15% A at 15−20 min, 15−50% A at 20−50 min, and 50−100% A at 50−55 min. The UV−vis spectra were recorded from 200 to 500 nm.

Humidity Environment. Relative humidity (RH) was measured using an Amprobe digital thermohygrometer (Miramar, FL). A (65−75) ± 3% humidity environment was created with a saturated solution of potassium nitrate and ammonium chloride in a sealed desiccator. The ambient RH was measured daily and did not exceed 30 ± 3% in the laboratory or 0% in the chamber at 50 °C (**Table 1**).

Table 1. Sample Preparation for Stability Test

sample	temp (°C)	RH (%)	time	condition
G-1	room	0−30	0−9 weeks	room condition
G-2	room	65−75	0−9 weeks	in a desiccator
G-3	50	0	0−9 weeks	in an incubator
G-4	50	65−75	0−9 weeks	in a desiccator
G-5	50	60−90	0−9 weeks	in a desiccator in vacuo
G-6	100	100	1−4 h	reflux in water
G-7	100	0	1−4 h	heated in an oven

Chemicals and Reagents. HPLC grade acetonitrile (J. T. Baker, Phillipsburg, NJ) and methanol (E. Merck, Darmstadt, Germany) were used for sample preparation and HPLC analysis. Reagent grade formic acid (purity > 98%, E. Merck) was used in this study.

Standards. Cimiracemoside F (**1**) and 23-epi-26-deoxyactein (**2**) (formerly known as 27-deoxyactein, 60.74% purity) were purchased from ChromaDex (Santa Ana, CA). Caffeic acid (**3**) and ferulic acid (**4**) were purchased from Sigma Chemical Co. (St. Louis, MO). Isoferulic acid (**5**) (97.37% purity), fukinolic acid (**6**) (95.90% purity), cimicifugic acid A (**7**) (96.95% purity), and cimicifugic acid B (**8**) (93.74% purity) were isolated from black cohosh extract (**Figure 1**). The methods to isolate compounds **5**−**8** were described in our previous publications (*30, 31*).

Black Cohosh Samples. Black cohosh roots and rhizomes and black cohosh ethanolic extract were supplied by PureWorld Botanicals Inc. (Naturex) (South Hackensack, NJ). Black cohosh encapsulated extract and excipients (maltodextrin and tricalcium phosphate) were provided by the Research Pharmacy of Columbia University Medical Center; each capsule contained 40 mg of powdered black cohosh ethanolic extract (PureWorld). The capsules from four different bottles were mixed and randomly grouped before the study.

Each black cohosh sample [the roots and rhizomes (ca. 1 g), the extract (400−800 mg for triterpene glycosides and ca. 80 mg for polyphenols), or the encapsulated material (12 capsules)] was put in a 20 mL scintillation vial for storage in the experiments. The stability study of triterpene glycosides was based on the black cohosh extract only.

Sample Storage and Treatment. Five groups (G-1−G-5) were prepared for study. Each group was composed of three kinds of black cohosh samples: root and rhizome, extract, and capsules. G-1 was stored at room temperature (20−25 °C) and humidity (≤30%); G-2 and G-4 were both stored at 65−75% RH. The former was kept at room temperature, and the latter was stored at 50 °C; G-3 was kept at 50 °C and a relatively dry condition; G-5 was kept in a sealed container with more than 75% RH at 50 °C under vacuum. The extracts in G-1, G-3, and G-4 were used for the stability test of both triterpene glycosides

and polyphenols. The other samples were used for the stability study of polyphenols only.

Two groups of black cohosh extract (G-6 and G-7) were used for additional stability tests of polyphenols. The samples of G-6 were refluxed in water for 1 and 4 h; the samples of G-7 were directly heated at 100 °C in an oven for 1 and 4 h (**Table 1**).

Three pure polyphenols were employed for the stability study: caffeic acid (**3**), ferulic acid (**4**), and fukinolic acid (**6**). These compounds were stored at two different conditions with samples G-3 and G-4. These polyphenols were also used for the stability test under direct heating and refluxing conditions.

Sample Preparation for Analysis. *Black Cohosh Extract.* Extract (400−800 mg) was suspended in 50 mL of 0.5% NaOH water solution. The solution was sonicated for 5 min and then transferred into a 120 mL separatory funnel and partitioned with chloroform (50 mL) by shaking the funnel vigorously for 2 min. The resulting NaOH aqueous solution was partitioned three more times using 50 mL of chloroform each time. All chloroform fractions were combined and evaporated under reduced pressure. The resulting residue was dissolved in 2 mL of DMSO and filtered through a 0.45 μm nylon membrane. The filtered sample was then analyzed for triterpene glycosides by HPLC-PDA.

The extract (ca. 80 mg) was dissolved in 70% MeOH (10 mL) and sonicated for 5 min. The resulting solution was filtered through a 0.45 μm membrane filter for HPLC analysis of the polyphenols. For the sample from G-6, methanol was added to the aqueous solution to achieve 70% methanol and then analyzed by HPLC-PDA.

Black Cohosh Capsule. The powder from 10 capsules was dissolved with 70% MeOH (50 mL) in a sealed bottle and sonicated for 10 min. The resulting solution was filtered through a 0.45 μm membrane filter for HPLC analysis. The efficiency of the extraction method was further evaluated as follows: the powder from 10 capsules was placed into a 50 mL plastic centrifugal tube and extracted with 50 mL of 70% MeOH. The tube was sealed and sonicated for 5 min and then centrifuged for 10 min at 3200 rpm. After the solution was removed, the residue was further extracted according to the same method two more times. The resulting solutions were combined and dried under vacuum at 25−30 °C. The residue was dissolved with 70% MeOH (50 mL). The solution was passed through a 0.45 μm membrane filter for HPLC analysis of the polyphenols.

Black Cohosh Roots and Rhizomes. Dried roots and rhizomes (ca. 1 g) were ground and extracted with 70% MeOH (25 mL), sonicated for 5 min, and centrifuged for 10 min. After the supernatant was decanted, the residue was further extracted according to the same method two more times. The resulting solutions were combined and dried under vacuum at 25−30 °C. The residue was dissolved with 25 mL of 70% MeOH in a volumetric flask and filtered through a 0.45 μm membrane filter for HPLC analysis of the polyphenols.

Sample Analysis. The black cohosh samples stored at different conditions, as well as the three pure polyphenols, were analyzed by HPLC-PDA at 0, 3, 6, and 9 weeks. For the quantitative analysis, the amounts of three triterpene glycosides, **1**, **2**, and actein, and six polyphenols, **3**−**8**, in the samples were measured. For the qualitative analysis, the constituents were identified by comparison of their UV properties and HPLC retention times with those of the standard compounds.

Validation of HPLC Method. Validation of this assay was in compliance with the AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (*32*). The method was validated with respect to linearity, recovery, and sensitivity.

Triterpene Glycosides. Seven stock solutions of standards **1**−**2** were prepared in DMSO with concentrations between 12.5 and 4000 μg/mL. Calibration curves were established on six data points. The limit of detection (LOD) and the limit of quantitation (LOQ) were determined on the basis of signal-to-noise ratios of about 3:1 and 10:1, respectively.

Polyphenols. Six stock solutions containing about 1 mg/mL of standards **3**−**8** were prepared independently in 70% MeOH. For calibration purposes, working solutions were freshly prepared by diluting each stock solution with 70% MeOH. Calibration curves were established on six data points covering the concentrations of 0.3−330 μg/mL. Each calibration curve was obtained by plotting the peak area of the standard at each level prepared versus the concentration of the standard.

Interday precision and accuracy were evaluated by performing three injections of standard mixture solutions at two concentration levels for three consecutive days. Intraday precision and accuracy were determined with three injections of a standard mixture solution at two concentration levels over a 1 day period. The precision at each concentration was expressed as the percentage relative standard deviation (RSD, %) of the measured concentration of standards, whereas the accuracy was assessed for each standard and expressed as percent relative error (RE, %) by comparing the nominal concentration with measured concentration.

Six standard compounds **3**−**8** were used for the recovery study of the native extract and the black cohosh capsule material, whereas three polyphenols, caffeic acid (**3**), ferulic acid (**4**), and fukinolic acid (**6**), were chosen for the recovery study of black cohosh roots and rhizomes. The recovery for the extraction of the roots and rhizomes was achieved by adding known amounts of the standards (ca. 2 mg each) to the ground rhizomes and roots of *A. racemosa* (1 g) prior to extraction. The recoveries for the extract and capsule were conducted by adding about 0.2 mg from a stock solution (0.167 mg/mL of each standard) to the samples before extraction (the amounts of compounds **6**−**8** used for the recovery experiment in the capsule were 7.8, 1.5, and 2.8 mg, respectively). The recovery was determined by subtracting the values obtained for the control matrix preparation from the sample added with standards, divided by the amount of standards, and expressed as percentage. LOD and LOQ were calculated as signal-to-noise ratios with minimal values of 3:1 and 10:1, respectively.

RESULTS AND DISCUSSION

Qualitative Analysis. When black cohosh extract was refluxed with water for 1 h, three fukiic acid ester derivatives, fukinolic acid (**6**) and cimicifugic acids A (**7**) and B (**8**), decomposed significantly; these three polyphenols decomposed almost completely after 4 h of refluxing (**Figure 2B**,**C**). This change can be explained by hydrolysis of the three fukiic acid ester derivatives; as the three fukiic acid ester derivatives decomposed, the amounts of three hydroxycinnamic acid derivatives, caffeic acid (**3**), ferulic acid (**4**), and isoferulic acid (**5**), significantly increased, suggesting that the fukiic acid ester derivatives in black cohosh were unstable and easily hydrolyzed. Over time, as the amount of fukiic acid ester derivatives decreased, the amounts of hydroxycinnamic acid derivatives (**3**−**5**) did not increase, likely because they, in turn, were oxidized.

When black cohosh extract was heated at 100 °C, at first, the fukiic acid ester derivatives decomposed mainly by hydrolysis, likely due to trace amounts of water in the extract and, therefore, the amount of isoferulic acid (**5**) increased (**Figure 2A**.**D**). However, when the water in the extract evaporated, hydrolysis stopped and oxidation became the major reaction to cause the decomposition of the polyphenols. Because antioxidant capacities of polyphenols in black cohosh differed and isoferulic acid (**5**) showed the weakest antioxidant activity among the major polyphenols in black cohosh (*19, 21*), isoferulic acid (**5**) became the major phenolic constituent (**Figure 2E**) in the extract after heating for 4 h at 100 °C.

Studies on standard compounds showed similar results regarding the stability of these polyphenols. When fukinolic acid (**6**) was refluxed with water, significant hydrolysis was observed, and **6** almost completely decomposed in 4 h. Meanwhile, caffeic acid (**3**) and some unidentified constituents were generated and/or increased (**Figure 3A**−**C**). The decomposition of ferulic acid (**4**) was different from that of fukinolic acid. Because **4** could not be hydrolyzed when it was refluxed with water, oxidation of the side chain on the benzene ring became the major reaction for this compound. As a result, degraded constituents from **4**

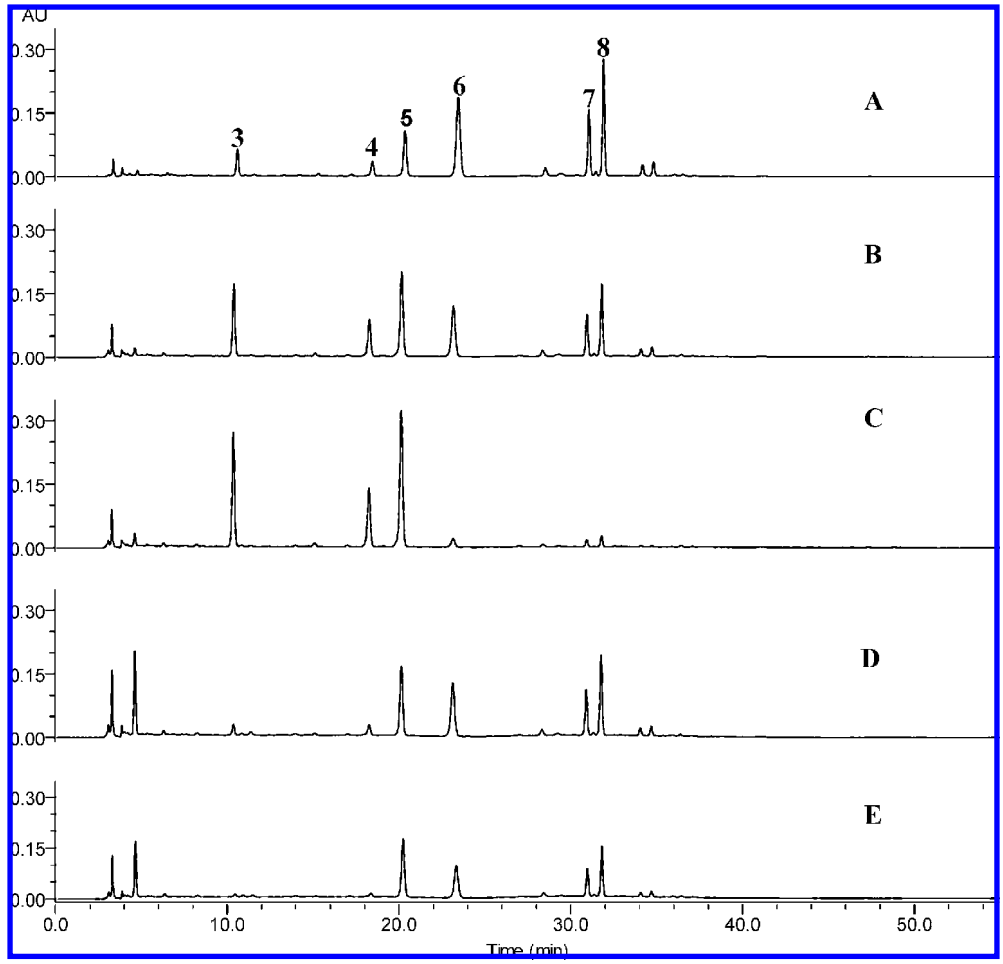

Figure 2. Major polyphenols in black cohosh extract and in experimental conditions (320 nm): (**A**) original extract; (**B**) reflux with water for 1 h; (**C**) reflux with water for 4 h; (**D**) heated at 100 °C for 1 h; (**E**) heated at 100 °C for 4 h.

were produced, as observed at 12−13 and 35−36 min on the HPLC chromatograms (**Figure 3D−F**).

When caffeic acid (**3**) and ferulic acid (**4**) were directly heated at 100 °C without water, there was no significant decomposition detected, suggesting that water is a key factor causing black cohosh polyphenols to decompose. Under open-air conditions, black cohosh cannot be kept completely dry, so atmospheric water can contribute to the decomposition of polyphenols. Also, water generated through the dehydrogenation reactions with compounds such as carbohydrates in the extract contribute to the decomposition. Therefore, it is reasonable that the hydroxycinnamic acid derivatives decompose more easily when they are present in an extract.

Quantitative Analysis. Black cohosh samples in G-1−G-5 stored at different conditions were analyzed for the amount of nine selected major constituents at 0, 3, 6, and 9 weeks. The results are shown in **Tables 2** and **3**. According to the analytical results, the triterpene glycosides were stable during the 9 week experiment. However, black cohosh polyphenols were stable at room temperature and ambient RH condition, but not at high temperature and high RH condition, indicating that the major polyphenols in black cohosh are sensitive to the humidity, especially at high temperature.

Black Cohosh Extract. The amounts of the three selected major triterpene glycosides in extracts G-1, G-3, and G-4 were quite similar over the 9 week study, indicating that black cohosh could be stored for a certain time without significant change in triterpene glycosides.

The major polyphenols in the extracts from G-1, G-2, and G-3 were not significantly different after 9 weeks of storage, but there was only 43% of the total polyphenolic content (TPC) remaining in the extract of G-4, suggesting that the major polyphenols in black cohosh were unstable under high temperature and high RH (**Table 3**). The main factor in the decrease in TPC content of the G-4 extract was most likely hydrolysis of the fukiic acid ester derivatives, which were the major polyphenols and accounted for 80−90% of TPC. In the extract from G-4, 65% of fukiic acid ester derivatives decomposed after storage for 9 weeks (**Figure 4A**). In the extract from G-5, due to a lower amount of air and a higher humidity, the hydrolysis of fukiic acid ester derivatives was even greater than that of any other sample. As a result, only 17% of fukiic acid ester derivatives could be detected in the resulting extract after 9 weeks. When the extract from G-6 was refluxed with water for 4 h, the amount of the fukiic acid ester derivatives in the resulting extract decreased to 10% of TPC (**Table 3**). Therefore, a dry environment is important to keep black cohosh polyphenols from changing significantly.

Black Cohosh Plant Material. Polyphenols in the plant samples showed stability similar to those in the extract samples (**Table 3**). The major polyphenols in the plant samples from G-1, G-2, and G-3 were stable, whereas TPC decreased significantly in G-4 at 9 weeks, but the decomposition rate of the major polyphenols in the plant samples was much lower than that in the extract samples. About 80% of TPC was detected in the resulting plant material after storage for 9 weeks at high

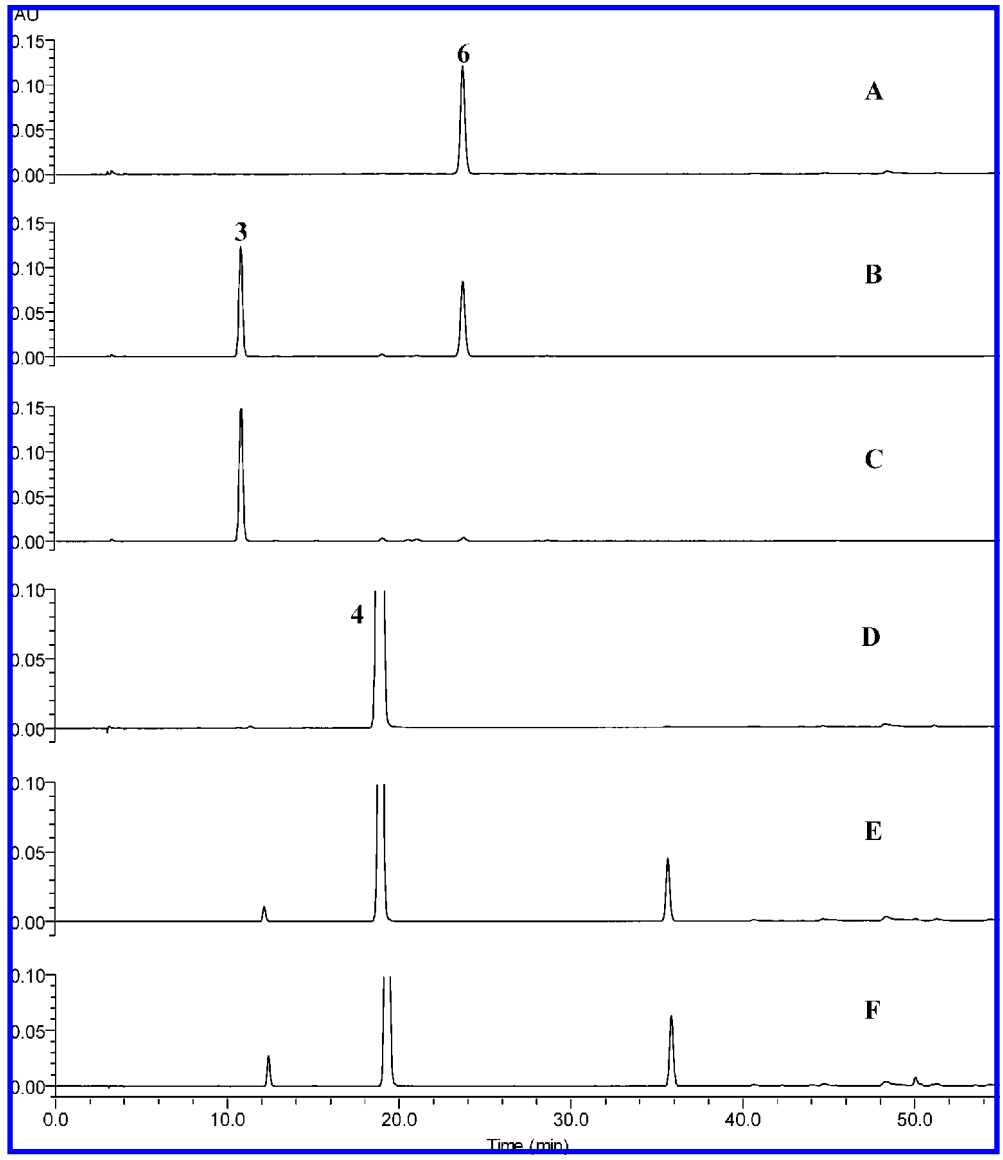

Figure 3. Decomposition of fukinolic acid (**6**) and ferulic acid (**4**) in water (280 nm). Fukinolic acid refluxing time: (**A**) 0 h; (**B**) 1 h; (**C**) 4 h. Ferulic acid refluxing time: (**D**) 0 h; (**E**) 4 h; (**F**) 8 h.

Table 2. Amount of the Three Triterpene Glycosides in the Black Cohosh Extract (Percent)[a]

sample	week	cimiracemoside F	actein	23-epi-26-deoxyactein	total
original	0	0.20 ± 0.007	0.34 ± 0.009	0.26 ± 0.015	0.80
G-1	9	0.19 ± 0.004	0.32 ± 0.012	0.25 ± 0.002	0.76
G-3	3	0.19 ± 0.002	0.34 ± 0.002	0.26 ± 0.006	0.78
	6	0.19 ± 0.005	0.32 ± 0.011	0.26 ± 0.011	0.77
	9	0.19 ± 0.003	0.33 ± 0.007	0.26 ± 0.003	0.78
G-4	3	0.18 ± 0.012	0.33 ± 0.006	0.26 ± 0.008	0.77
	6	0.18 ± 0.003	0.32 ± 0.003	0.26 ± 0.009	0.76
	9	0.19 ± 0.001	0.35 ± 0.009	0.26 ± 0.004	0.80

[a] Data are the mean ± SD of triplicate determination.

temperature and humidity conditions (**Figure 4B**), indicating that black cohosh, in the form of ground whole plant material, helped to prevent the decomposition of the polyphenols.

Black Cohosh Capsules. The polyphenolic profile of black cohosh capsules is similar to that of the plant material with regard to the number of different polyphenols, but the ratios of these compounds are significantly different from common black cohosh extracts/products. Instead of fukiic acid ester derivatives, isoferulic acid (**5**) was determined to be the major polyphenolic constituent in the capsule. Because **5** is more stable than most other polyphenols in black cohosh with regard to both oxidation and hydrolysis, after 9 weeks of storage at high temperature and high RH conditions, almost 90% of isoferulic acid (**5**) was determined to be unchanged. Most other polyphenols in the capsule were also only slightly reduced, possibly due to the protection by the capsule coating and the excipients. The TPC in the capsule was reduced only 16% as compared with 57% in the extract after storage for 9 weeks at high temperature and high RH condition (**Table 3**).

Validation of HPLC Method. The method for the quantitative analysis of polyphenols by HPLC-PDA was validated with respect to linearity, recovery, and sensitivity. Linear regression analysis for each standard was performed by the external standard method. Good linearity of six-point calibration curves was obtained for all standards between peak area and concentration ($r^2 = 1.0000$) over the range test (ca. 0.3−330 μg/mL).

Intra- and interday analyses of the same solution containing triterpene glycosides **1** and **2** or polyphenols **3**−**8** in two different concentrations (triterpene glycoside, 0.25 and 1.00 mg/mL; polyphenol, 1.67 and 167 μg/mL) were used to validate the precision and accuracy of the method. The precision was calculated as RSD (percent). The RSD of the standards **1** and

Table 3. Total Amount of the Six Major Polyphenolic Acids in the Black Cohosh Samples (Percent)[a]

sample	week	caffeic	ferulic	isoferulic	fukinolic	Cimi-A	Cimi-B	total
extract	0	0.15 ± 0.002	0.10 ± 0.003	0.35 ± 0.008	2.60 ± 0.063	0.86 ± 0.017	1.47 ± 0.026	5.54
G-1	3	0.17 ± 0.002	0.11 ± 0.000	0.39 ± 0.001	2.68 ± 0.007	0.93 ± 0.000	1.58 ± 0.001	5.86
	6	0.17 ± 0.003	0.11 ± 0.002	0.37 ± 0.001	2.58 ± 0.007	0.89 ± 0.000	1.52 ± 0.002	5.63
	9	0.16 ± 0.002	0.11 ± 0.001	0.35 ± 0.003	2.53 ± 0.007	0.88 ± 0.004	1.50 ± 0.006	5.53
G-2	3	0.16 ± 0.001	0.11 ± 0.000	0.39 ± 0.001	2.64 ± 0.027	0.92 ± 0.008	1.56 ± 0.013	5.77
	6	0.16 ± 0.002	0.11 ± 0.002	0.37 ± 0.001	2.53 ± 0.001	0.88 ± 0.001	1.50 ± 0.002	5.55
	9	0.16 ± 0.003	0.11 ± 0.004	0.36 ± 0.008	2.44 ± 0.004	0.85 ± 0.002	1.45 ± 0.003	5.38
G-3	3	0.16 ± 0.002	0.10 ± 0.002	0.38 ± 0.008	2.61 ± 0.066	0.91 ± 0.022	1.54 ± 0.035	5.69
	6	0.14 ± 0.001	0.10 ± 0.000	0.36 ± 0.001	2.53 ± 0.013	0.88 ± 0.007	1.51 ± 0.011	5.52
	9	0.14 ± 0.004	0.10 ± 0.002	0.35 ± 0.003	2.48 ± 0.041	0.86 ± 0.007	1.48 ± 0.014	5.41
G-4	3	0.12 ± 0.002	0.09 ± 0.002	0.47 ± 0.004	1.73 ± 0.007	0.63 ± 0.001	1.08 ± 0.003	4.12
	6	0.11 ± 0.003	0.08 ± 0.000	0.53 ± 0.009	1.17 ± 0.070	0.44 ± 0.022	0.78 ± 0.035	3.11
	9	0.07 ± 0.001	0.06 ± 0.001	0.54 ± 0.005	0.82 ± 0.011	0.32 ± 0.001	0.57 ± 0.003	2.37
G-5	9	0.09 ± 0.004	0.06 ± 0.001	0.62 ± 0.009	0.41 ± 0.024	0.16 ± 0.009	0.28 ± 0.015	1.62
G-6	1h	0.41 ± 0.001	0.25 ± 0.001	0.63 ± 0.005	1.66 ± 0.007	0.53 ± 0.002	0.89 ± 0.004	4.39
	4h	0.65 ± 0.001	0.40 ± 0.001	1.02 ± 0.003	0.29 ± 0.002	0.09 ± 0.001	0.14 ± 0.000	2.59
G-7	1h	0.06 ± 0.001	0.07 ± 0.001	0.50 ± 0.003	1.69 ± 0.001	0.56 ± 0.005	0.96 ± 0.003	3.84
	4h	0.02 ± 0.001	0.03 ± 0.001	0.53 ± 0.002	1.31 ± 0.003	0.44 ± 0.002	0.77 ± 0.002	3.11
plant	0	0.02 ± 0.000	0.02 ± 0.000	0.09 ± 0.000	0.47 ± 0.001	0.14 ± 0.000	0.29 ± 0.000	1.10
G-1	3	0.02 ± 0.000	0.02 ± 0.000	0.09 ± 0.000	0.50 ± 0.000	0.14 ± 0.003	0.30 ± 0.000	1.07
	6	0.02 ± 0.000	0.02 ± 0.000	0.09 ± 0.000	0.51 ± 0.008	0.15 ± 0.001	0.30 ± 0.003	1.07
	9	0.02 ± 0.000	0.02 ± 0.000	0.09 ± 0.000	0.44 ± 0.001	0.13 ± 0.000	0.27 ± 0.001	0.98
G-2	3	0.02 ± 0.000	0.02 ± 0.000	0.09 ± 0.001	0.51 ± 0.002	0.15 ± 0.001	0.30 ± 0.001	1.09
	6	0.02 ± 0.002	0.02 ± 0.000	0.09 ± 0.001	0.49 ± 0.004	0.14 ± 0.001	0.30 ± 0.002	1.07
	9	0.02 ± 0.001	0.02 ± 0.000	0.09 ± 0.000	0.47 ± 0.001	0.14 ± 0.001	0.28 ± 0.001	1.02
G-3	3	0.02 ± 0.000	0.02 ± 0.000	0.09 ± 0.000	0.43 ± 0.001	0.13 ± 0.001	0.27 ± 0.001	0.98
	6	0.02 ± 0.000	0.02 ± 0.001	0.10 ± 0.000	0.52 ± 0.001	0.15 ± 0.000	0.31 ± 0.001	1.13
	9	0.02 ± 0.000	0.02 ± 0.000	0.09 ± 0.000	0.47 ± 0.001	0.14 ± 0.000	0.29 ± 0.000	1.05
G-4	3	0.02 ± 0.000	0.02 ± 0.001	0.10 ± 0.000	0.47 ± 0.004	0.14 ± 0.001	0.29 ± 0.003	1.05
	6	0.02 ± 0.001	0.02 ± 0.000	0.11 ± 0.001	0.48 ± 0.003	0.14 ± 0.001	0.30 ± 0.002	1.06
	9	0.02 ± 0.001	0.02 ± 0.000	0.11 ± 0.000	0.36 ± 0.001	0.11 ± 0.001	0.24 ± 0.001	0.86
G-5	9	0.02 ± 0.000	0.02 ± 0.000	0.12 ± 0.001	0.32 ± 0.000	0.10 ± 0.000	0.22 ± 0.000	0.80
capsule	0	0.04 ± 0.001	0.03 ± 0.000	0.30 ± 0.004	0.25 ± 0.012	0.13 ± 0.003	0.26 ± 0.007	1.02
G-1	3	0.04 ± 0.000	0.04 ± 0.001	0.30 ± 0.005	0.22 ± 0.017	0.12 ± 0.006	0.24 ± 0.012	0.96
	6	0.04 ± 0.002	0.03 ± 0.001	0.30 ± 0.003	0.24 ± 0.007	0.13 ± 0.003	0.26 ± 0.004	0.99
	9	0.04 ± 0.001	0.04 ± 0.000	0.29 ± 0.003	0.21 ± 0.001	0.12 ± 0.001	0.23 ± 0.001	0.93
G-2	3	0.04 ± 0.000	0.04 ± 0.000	0.30 ± 0.001	0.22 ± 0.001	0.12 ± 0.001	0.24 ± 0.000	0.95
	6	0.04 ± 0.001	0.03 ± 0.001	0.29 ± 0.007	0.25 ± 0.003	0.13 ± 0.000	0.26 ± 0.002	1.01
	9	0.04 ± 0.002	0.04 ± 0.002	0.30 ± 0.018	0.24 ± 0.006	0.13 ± 0.001	0.26 ± 0.002	1.00
G-3	3	0.04 ± 0.004	0.04 ± 0.002	0.30 ± 0.010	0.22 ± 0.001	0.12 ± 0.002	0.24 ± 0.002	0.96
	6	0.03 ± 0.008	0.03 ± 0.005	0.30 ± 0.016	0.25 ± 0.015	0.13 ± 0.008	0.26 ± 0.016	1.00
	9	0.03 ± 0.008	0.03 ± 0.005	0.29 ± 0.010	0.22 ± 0.019	0.12 ± 0.008	0.24 ± 0.014	0.94
G-4	3	0.03 ± 0.000	0.03 ± 0.002	0.28 ± 0.020	0.21 ± 0.010	0.12 ± 0.003	0.23 ± 0.006	0.91
	6	0.03 ± 0.002	0.03 ± 0.001	0.24 ± 0.004	0.22 ± 0.008	0.12 ± 0.003	0.24 ± 0.005	0.86
	9	0.03 ± 0.000	0.03 ± 0.001	0.27 ± 0.020	0.20 ± 0.019	0.11 ± 0.006	0.22 ± 0.015	0.86
G-5	9	0.03 ± 0.000	0.03 ± 0.000	0.21 ± 0.001	0.18 ± 0.004	0.10 ± 0.002	0.20 ± 0.004	0.74

[a] Data are the mean ± SD of triplicate determinations.

2 varied between 0.36 and 3.93% ($n = 3$) on the same day, but from 0.37 to 2.54% ($n = 9$) on different days. Meanwhile, the RSD of the standards **3**−**8** varied between 0.41 and 2.44% ($n = 3$) on the same day and from 0.35 to 2.45% ($n = 9$) on different days. The accuracy was calculated as RE (percent). The RE for the standards **1** and **2** ranged from −2.32 to 2.72% ($n = 3$) on the same day and from −2.22 to 2.38% ($n = 9$) between days, whereas the RE for the standards **3**−**8** varied from −1.53 to 1.84% ($n = 3$) on the same day and from −3.07 to 3.07% ($n = 9$) between days.

The recoveries (percent) for caffeic acid (**3**), ferulic acid (**4**), and fukinolic acid (**6**) from the plant material were 105.6, 99.9, and 95.6%, respectively. Similar recoveries for closely related compounds would be expected. The recoveries (percent) for the polyphenols **3**−**8** from the extract were 105.6, 104.1, 108.6, 120.0, 105.0, and 111.5%, respectively. However, the recoveries from the capsule for **3**−**8** were 93.7, 99.1, 103.2, 62.1, 71.5, and 70.1%, respectively.

The LOD and LOQ of the six polyphenols were established by means of the baseline noise method. The LODs for these compounds were found to be in the range of 66−134 ng/mL, whereas the LOQs were in the range of 310−334 ng/mL.

Evaluation of the Method for the Capsule Extraction. The capsules used for this study were made from black cohosh ethanolic extract with an excipient. To extract the black cohosh compounds from the contents of the capsule, we used 70% MeOH once. The efficiency of this extraction method was evaluated. The total amount of the six polyphenols from the one-time extraction of the capsule was determined to be 1.02% (±0.019, $n = 4$), whereas it was 1.09% (±0.015, $n = 3$) from the three-time extraction, suggesting that >93% of the polyphenols was extracted from the capsule in the one-time extraction. After three extractions, the amount of polyphenols in the residue was determined to be 0.002−0.004%.

The black cohosh capsules we examined showed a somewhat different chemical profile with respect to the major polyphenols, compared to most chemical profiles of black cohosh plant

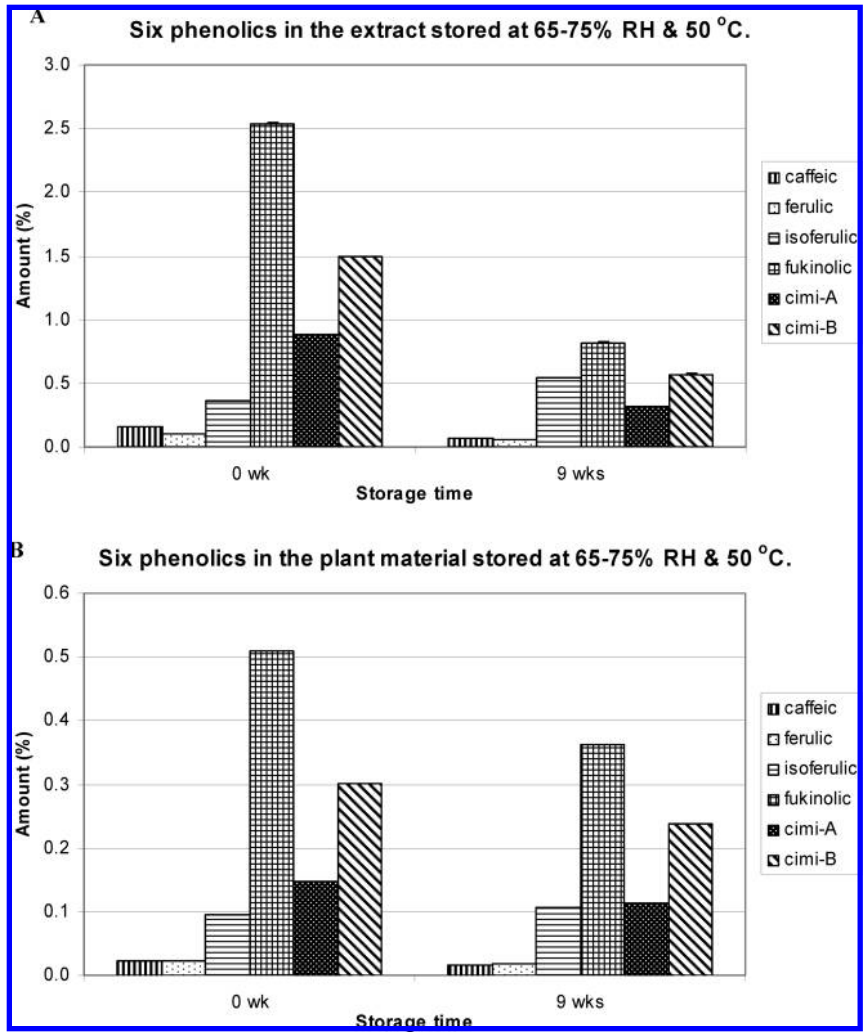

Figure 4. Changes in the amounts of the six major polyphenols in black cohosh extract (**A**) and plant material (**B**) after storage in a high relative humidity and temperature condition.

material, extracts, and products. The total amount of the three fukiic acid ester derivatives in the capsules was between 60 and 66% among the six major polyphenols, whereas the amount of isoferulic acid (**5**) was greater than 28%. According to our previous study, when black cohosh plant material was extracted with different alcohols such as 80% methanol, 75% ethanol, and 40% isopropanol, the resulting extracts showed similar chemical profiles with respect to the major polyphenols (*31*). Therefore, the uncommon chemical profile for polyphenols of the tested capsules may be caused by other factors, such as excipients. In our recovery experiments, when 0.3−0.5 mg of the standards were used, satisfactory recoveries were achieved for compounds **3**−**5**, but the recoveries for **6**−**8** were 10, 39, and 35%, respectively. When the amounts of **6**−**8** were increased to 1.5−7.8 mg, the recoveries were still unexpectedly low (62−72%). Two excipients, maltodextrin and tricalcium phosphate, were used with the extract in this capsule. We conducted further experiments which demonstrated that tricalcium phosphate could selectively absorb fukiic acid ester derivatives, thus preventing these compounds from being extracted with aqueous alcohol. Our study showed that when 100 mg of 60% methanol extract of black cohosh was mixed with 2.5 g of tricalcium phosphate, the recoveries for **6**−**8** were 8−9%, whereas the recoveries for **3**−**5** were 93−96%. When the amount of the extract increased, or the content of tricalcium phosphate decreased, the recoveries of **6**−**8** increased due to the limits of absorption for tricalcium phosphate. However, on the basis of our study, because tricalcium phosphate is soluble in hydrochloric acid, this enables it to release these absorbed compounds into solution. Therefore, absorption of **6**−**8** by tricalcium phosphate may not affect the bioactivities of the capsule due to the existence of hydrochloric acid in the human stomach. Therefore, when polyphenols from black cohosh products that contain tricalcium phosphate are analyzed, proper extraction techniques would be critical for accurate quantitative analysis.

Effect of Temperature on Selected Marker Compounds. In 2006, Mukhopadhyay et al. (*20*) attempted to optimize a method to extract phenolic acids from black cohosh, and they found that the TPC in the extract increased with temperature up to 90 °C. They concluded that 90 °C was the optimum temperature for maximum recovery of phenolics from black cohosh (*20*). The phenolic acids they selected for the TPC, caffeic acid (**3**), ferulic acid (**4**), sinapic acid, and isoferulic acid (**5**), were all hydroxycinnamic acid derivatives, which represent a small portion of the TPC of black cohosh. Because hydroxycinnamic acid derivatives cannot hydrolyze, these conclusions cannot be transferred to the major polyphenolic constituents (**6**−**8**) of black cohosh, which are hydrolyzable. According to our study, fukiic and picidic ester derivatives in black cohosh were not stable at high temperature and RH condition; thus, the extraction of these compounds at 90 °C is not appropriate for this class of compounds. Because the hydrolysis of these polyphenols in black cohosh could give hydroxycinnamic acid

derivatives and therefore lead to the increased yields of caffeic acid (**3**), ferulic acid (**4**), and isoferulic acid (**5**), it is reasonable that Mukhopadhyay et al. found that high temperature is helpful in obtaining more polyphenols. However, caution is needed because a method that gives high yields of polyphenols may not necessarily provide a full range of phenolic constituents from black cohosh.

Possible Benefit of Polyphenol Decomposition. A previous study indicated that isoferulic acid (**5**) had in vivo antihyperglycemic activity and could enhance glucose utilization in vivo (*33*). According to our study, isoferulic acid (**5**) was one of the major polyphenols in black cohosh, and its amount was between 0.04 and 0.10% (w/w) in plant material and between 0.05 and 0.35% in the extract. However, because isoferulic acid (**5**) is not affected by hydrolysis, and its antioxidant activity is much lower than other polyphenols in black cohosh due to the *p*-methoxyl group on the structure (*19, 21*), the relative amount of this compound in black cohosh/products could be controlled and increased by special processing. According to **Table 3**, when the amounts of other polyphenols dropped during storage, the content of isoferulic acid (**5**) increased in G-4 and G-5 for both extract and plant samples after a 9 week storage at high temperature and humidity (**Figure 4**). The highest amount was 0.62% in the extract sample (G-5). Moreover, if the black cohosh extract was further processed by refluxing in water for 4 h, the amount of isoferulic acid (**5**) was greater than 1% in the extract. Therefore, isoferulic acid (**5**) can become the richest polyphenol in the black cohosh extract, and this may be of interest to those studying isoferulic acid for diabetes (*33*).

Antioxidant and Biological Activity. Wojcikowski et al. (*24*) investigated the antioxidant capacity of 55 medicinal herbs used to treat the urinary system. Using the oxygen radical absorbance capacity (ORAC) method, they found that black cohosh was one of the plants that showed the strongest antioxidant activity; the total ORAC value of black cohosh was 1264.95 μmol of TE/g of dw (*22*). This suggests that as antioxidant constituents, the polyphenols in black cohosh may be more easily oxidized than those in other plants. Black cohosh, therefore, needs to be more carefully stored to reduce changes in its unstable polyphenolic constituents. Many polyphenols in black cohosh have known biological activity. For example, caffeic acid (**3**), ferulic acid (**4**), and fukinolic acid (**6**) were found to reduce menadione-induced DNA damage in cultured S30 breast cancer cells (*21*), whereas the phenylpropanoid ester 3,4-dihydroxyphenacyl caffeate was reported to inhibit proliferation of LNCaP human prostate carcinoma cells (*17*). Therefore, any change of constituents could result in a change of the biological activity of black cohosh. Thus, it is important to study the stability of black cohosh polyphenols to ensure the efficacy, and perhaps safety, of black cohosh.

Synergy and Menopausal Symptoms. The relevant constituents and mechanism of black cohosh responsible for relieving menopausal symptoms are still unknown. Whether black cohosh is of benefit to women's health and for what conditions remains open for further investigation (*2, 7, 34, 35*). Most previous studies have indicated that black cohosh does not contain estrogen-like constituents (*36*−*38*). Because menopausal symptoms are diverse and involve many organ systems, the possibility exists that black cohosh may work for one or more menopausal symptoms through complex interactions. Black cohosh may work via a single compound, multiple compounds, or perhaps by synergetic action of several compounds (*39*−*41*). Because synergetic mechanisms would depend on a group of constituents instead of a single compound, the chemical profile of these constituents may be critical to the biological activities, and any change in the chemical profile may alter biological response.

Many fukiic and picidic ester derivatives in black cohosh, such as cimicifugic acids A−G, were reported from *Actaea* (*Cimicifuga*) species only and are unique to the genus *Actaea*. However, because these compounds often show stronger antioxidant activity than many other polyphenols in black cohosh (*19, 21*) and they are also easily hydrolyzed according to our study, these constituents are likely to be more unstable and more easily decomposed by hydrolysis and oxidation. Considering that black cohosh extract/products are often produced by a variety of methods, some unstable constituents such as the polyphenols may have very different chemical profiles in the resulting extract/products compared with the original plant material, and this may affect biological activity. Thus, care should be taken in interpreting biological activity when black cohosh products are compared, due to potentially different chemical profiles caused by the stability or instability of the constituents.

In conclusion, the triterpene glycosides from black cohosh are stable. The polyphenolic constituents are stable only at room temperature and low humidity and are not stable at higher temperature and/or humidity due to hydrolysis and/or oxidation. The chemical profile of black cohosh extracts/products may change with the method of production and storage conditions, due to decomposition of the unstable polyphenolic constituents.

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