

Analysis of formononetin from black cohosh (*Actaea racemosa*)

B. Jiang^a, F. Kronenberg^a, M.J. Balick^b, E.J. Kennelly^{c,*}

^aThe Richard and Hinda Rosenthal Center for Complementary & Alternative Medicine, Department of Rehabilitation Medicine, College of Physicians & Surgeons, Columbia University, New York, USA

^bInstitute of Economic Botany, The New York Botanical Garden, Bronx, New York, USA

^cDepartment of Biological Science, Lehman College and The Graduate Center, City University of New York, Bronx, New York, USA

Received 13 April 2005; accepted 7 June 2005

Abstract

Black cohosh has been widely used as an herbal medicine for the treatment of symptoms related to menopause in America and Europe during the past several decades, but the bioactive constituents are still unknown. Formononetin is an isoflavone with known estrogen-like activity. This compound was first reported to be isolated from black cohosh in 1985, but subsequent research in 2002 using HPLC-PDA and LC-MS revealed no evidence to show the presence of formononetin in 13 populations of American black cohosh. A more recent report published in 2004 claimed to detect formononetin in an extract of black cohosh rhizomes using a TLC-fluorescent densitometry method. To further resolve these conflicting reports, we analyzed black cohosh roots and rhizomes for the presence of formononetin, using a combined TLC, HPLC-PDA and LC-MS method. We examined both methanolic and aqueous methanolic black cohosh extracts by HPLC-PDA and LC-MS methods, and did not detect formononetin in any extracts. We further determined the limits of detection of formononetin by HPLC-PDA and LC-MS. Our experimental results indicated that the sensitivity and accuracy of the HPLC-PDA and LC-MS methods for the analysis of formononetin were slightly higher than those of the reported fluorescent method, suggesting that the HPLC-PDA and LC-MS methods were reliable for the analysis of formononetin from black cohosh. We also repeated the reported TLC method to concentrate two fractions from a modern black cohosh sample and an 86-year-old black cohosh sample, respectively, and then analyzed these two fractions for formononetin using the HPLC-PDA and LC-MS method instead of the fluorescent method. Formononetin was not detected by HPLC-PDA or LC-MS. From the results of the present study it is not reasonable to attribute the estrogen-like activity of black cohosh extracts to formononetin.

© 2005 Elsevier GmbH. All rights reserved.

Keywords: Formononetin; Black cohosh; *Actaea racemosa*; *Cimicifuga racemosa*; TLC; HPLC-PDA; LC-MS; Analysis

Introduction

The roots and rhizomes of *Actaea racemosa* L. (black cohosh) (syn. *Cimicifuga racemosa*) have a long and

*Corresponding author. Tel.: +1 718 960 1105;
fax: +1 718 960 8236.

E-mail address: edward.kennelly@lehman.cuny.edu
(E.J. Kennelly).

diverse history of medicinal use in North America. The plant was traditionally used by Native Americans and early colonists to treat a variety of conditions including general malaise, malaria, rheumatism, abnormalities in kidney function, sore throat and menstrual irregularities, and to assist with childbirth (Barton, 1798; Rafinesque, 1828). During the past 40 years, in North America and Europe, this plant has been used as an herbal medicine primarily for the treatment of symptoms related to menopause. However, the constituents responsible for this bioactivity are still unknown (Borrelli et al., 2003).

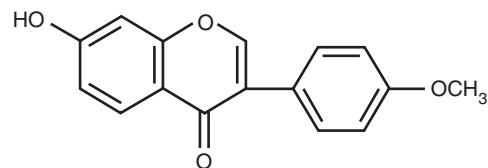
In a single report published in 1985, the isoflavonoid formononetin (**1**) was described from black cohosh (Jarry et al., 1985). Using the estrogen receptor assay as a pharmacological test system to detect the activity of different fractions, Jarry et al. found at least three different endocrine-active compounds in the MeOH extract of black cohosh rhizomes. After the chromatographic separations, one of the three compounds was identified as the isoflavone formononetin. The endocrine activity of this isoflavone was characterized in both described test systems, and formononetin was a competitor in the estrogen receptor assay, but did not reduce the serum levels of LH in ovariectomized rats (Jarry et al., 1985). This compound has since been repeatedly cited to explain the plant's observed estrogen-like effect.

In 2002, Kennelly et al. analyzed 13 populations of black cohosh collected in the eastern United States, as well as a commercially available black cohosh rhizome extract and a black cohosh product, Remifemin, for formononetin and ononin (formononetin-7-glucoside) by HPLC-PDA and LC-MS methods. Neither formononetin nor ononin was detected in any of the 13 plant populations, the commercial black cohosh extract or the black cohosh product (Kennelly et al., 2002).

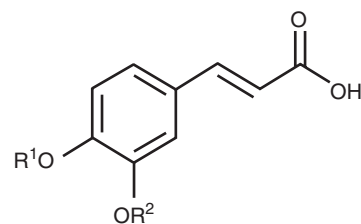
In 2004, Panossian et al. reported the detection of formononetin in black cohosh using a fluorescent TLC method. Formononetin was identified by its fluorescent property and quantitated by TLC-densitometry. The content of formononetin in the rhizomes was determined to be 0.00033% (3.3 ppm). The authors commented that formononetin was not detected by Kennelly et al. because the HPLC-PDA and LC-MS methods for the determination of formononetin were not sensitive enough for testing raw plant materials and drugs (Panossian et al., 2004). However, these authors do not report that they tried to repeat the methods of Kennelly et al.

It is imperative to evaluate whether formononetin is present in black cohosh because of its known bioactivity. We therefore decided to reexamine black cohosh roots and rhizomes for formononetin using HPLC-PDA, LC-MS and TLC methods, and to evaluate the

reliability of those reported analytical methods for the detection of formononetin.

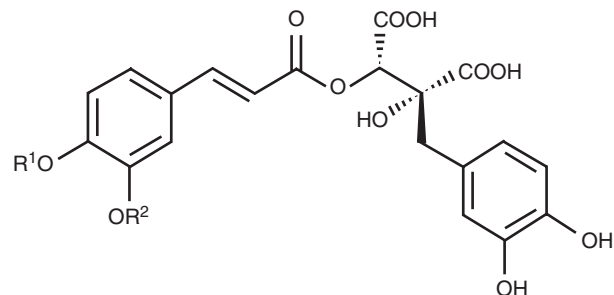


Formononetin (**1**)



Caffeic acid (**2**): $R^1 = H$ $R^2 = H$

Ferulic acid (**3**): $R^1 = H$ $R^2 = CH_3$



Fukinolic acid (**4**): $R^1 = H$ $R^2 = H$

Cimicifugic acid B (**5**): $R^1 = CH_3$ $R^2 = H$

Material and methods

General experimental procedures

The black cohosh samples and a formononetin standard (**1**) were analyzed using HPLC on a Waters 2695 separations module (Milford, USA) equipped with a 996 photodiode array detector (PDA), and operated with Empower software. Separations were carried out on a Phenomenex Aqua C_{18} column (4.6×250 mm, $5 \mu m$) at ambient temperature with a flow rate of 1.0 ml/min. The sample volume injected was 10 μl and data were analyzed at 258 nm. The mobile phase consisted of a step gradient starting with 5% (v/v) acetonitrile (solvent A) in 10% aqueous formic acid (B) and increasing to 100% acetonitrile over 55 min. The gradient profile was: 0–15 min: 5–15% A; 15–20 min: 15% A; 20–50 min: 15–50% A; 50–55 min: 50–100% A. The UV/vis spectra were recorded from 200 to 600 nm.

Mass spectra were recorded on a LCQ Mass Spectrometer (ThermoFinnigan, San Jose, USA) equipped with an electrospray ionization (ESI) source. ESI was performed with the spray voltage at 4.00 kV and the spray current at 0.49 μ A. The capillary voltage was set to -29.00 V, and the capillary temperatures were 230.0°C . The sheath gas and auxiliary gas, both nitrogen, had flow rates of 80 and 30 units, respectively. A mass range of 150–800 amu was scanned in the negative mode. Separations were carried out on a Waters 2690 HPLC equipped with a Phenomenex Aqua C_{18} column (4.6×250 mm, $5\mu\text{m}$) at ambient temperature with a flow rate of 0.5 ml/min.

Chemicals and reagents

HPLC grade acetonitrile (J.T. Baker, Phillipsburg, USA), methanol (Fisher Scientific, Fair Lawn, USA), and formic acid (E. Merck, Darmstadt, Germany) were used for sample preparation and HPLC and LC-MS analysis. Reagent grade ethyl acetate (EM Science, Gibbstown, USA), toluene (EM Science, Gibbstown, USA) and HPLC grade glacial acetic acid (J. T. Baker, Phillipsburg, USA) were used for the development of the TLC.

Standards

Formononetin (**1**) was purchased from Fluka Chemie in June 2004 (Lot&Filling code: 452969/1 25103086). The purity of **1** is stated to be higher than 99% (by TLC) on the label. The purity was further verified by our experiments. When **1** was analyzed at different concentrations, no impurities were observed by HPLC-PDA chromatograms of **1**.

Sample preparation

Black cohosh roots and rhizomes were supplied by PureWorld Botanicals Inc. (South Hackensack, USA) with lot # 9-2677 and item # 02580-001, and ground to a fine powder before being used in this study. In addition, a black cohosh sample, collected by Rusby in 1918 and stored in The New York Botanical Garden (NYBG), was analyzed for formononetin (**1**) in this study. The 86-year-old black cohosh was a commercial sample with an identification number of H.H. Rusby #4922.

Sample 1: Black cohosh roots and rhizomes (10.0601 g) were extracted with 100 ml of 80% MeOH/ H_2O at ambient temperature for 12 h and then filtered. The marc was re-extracted with 80% MeOH/ H_2O three more times. The filtrates were combined and evaporated in vacuo (at 45°C) to give a brown extract (1.5023 g). A part of the extract (7.46 mg) (sample 1-a) was dissolved

with 2 ml of 70% MeOH/ H_2O and subjected to HPLC-PDA and LC-MS analyses. A part of the extract (102.22 mg) (sample 1-b) was dissolved with MeOH (1 ml), and the solution was sonicated for 15 min. Aliquots (0.3 ml) of the resulting supernatant were filtered and analyzed by HPLC-PDA and LC-MS.

Sample 2: Black cohosh roots and rhizomes (10.0964 g) were extracted with MeOH (100 ml) under reflux for 1 h and then filtered. The resulting plant material was extracted with MeOH (100 ml each) two more times by the same extraction method. The filtrates were combined and evaporated in vacuo (at 40°C) to give a yellow extract (1.0585 g). A portion of the extract (51.08 mg) was dissolved with MeOH (1 ml). The solution was sonicated for 15 min and subjected for analysis by HPLC-PDA and LC-MS.

Sample 3: Black cohosh roots and rhizomes (10.0070 g) were mixed with 1.20 mg of formononetin (**1**). The mixture was extracted with 80% MeOH/ H_2O using the method mentioned in the preparation of **sample 1** to yield a brown extract (1.4950 g). Parts of the extract (10.98 mg and 9.98 mg) (samples 3-a and 3-b) were dissolved with 70% MeOH/ H_2O (2 ml and 100 ml) and analyzed using HPLC-PDA and LC-MS. A part of the extract (7.60 mg) (sample 3-c) was dissolved with MeOH (0.3 ml) and underwent preparative silica gel TLC (10×10 cm, $250\mu\text{m}$) for formononetin using the method described in **sample 4**, and the relevant fraction was dissolved in MeOH (200 μl) and analyzed by HPLC-PDA and LC-MS.

Sample 4: Black cohosh roots and rhizomes (5.1993 g) were extracted twice with MeOH (25 ml each) in an ultrasonic bath for 30 min. The combined extracts were filtered, evaporated to dryness, and the residue (289.4 mg) was dissolved 2.0 ml of MeOH and centrifuged at 3500 rpm for 15 min. Aliquots (1.0 ml) of the resulting supernatant were separated by preparative silica gel 60 F_{254} TLC (10×10 cm, $250\mu\text{m}$) with formononetin as an indicator beside the sample. According to the R_f value of the indicator (formononetin), the silica gel within the range of the R_f -formononetin ± 0.5 cm was scraped and collected. The silica gel powder was eluted with MeOH (5 ml). The MeOH solution was evaporated, and the residue was redissolved in MeOH (200 μl) and analyzed by HPLC-PDA and LC-MS.

Sample 5: Powdered 86-year-old black cohosh roots and rhizomes (1.0097 g) were extracted twice with MeOH (10 ml each) in an ultrasonic bath for 30 min. The combined extracts were filtered, evaporated to dryness, and the residue (23.4 mg) was dissolved in 0.5 ml of MeOH. The solution was separated by preparative silica gel 60 F_{254} TLC using the same method mentioned in **sample 4**. The resulting fraction from the preparative TLC was analyzed by HPLC-PDA.

TLC

Separations of samples 3-c, 4, and 5 were performed on silica gel 60 F₂₅₄ TLC (10 × 10 cm, 250 μm) (EMD, Darmstadt, Germany). The mobile phase used for the separation was ethyl acetate:toluene:glacial acetic acid (17.5:80:2.5, by volume) (Panossian et al., 2004). The plates were visualized under UV light at 254 nm.

Limits of detection and recovery test

Formononetin (**1**) (0.75 mg) was dissolved with methanol (4 ml) to yield a solution with a concentration of 0.1875 mg/ml, and this solution was further diluted with methanol into a series of five-fold solutions. Limits of detection of formononetin for HPLC-PDA and LC-MS were determined by analysis of the peak height versus the baseline noise at a signal to noise ratio of 3:1. The recovery of formononetin was determined by a spiking experiment: formononetin (1.20 mg) was mixed with black cohosh roots and rhizomes (10.0070 g) and the mixture was extracted with 80% MeOH/H₂O (100 ml × 4, 12 h each time) at ambient temperature to yield a brown extract (1.4950 g). Parts of the extract (10.98 mg and 19.15 mg) were dissolved with 70% MeOH/H₂O (2 and 3 ml) and analyzed using HPLC-PDA and LC-MS to determine the recovery of formononetin. Based on this spiking experiment, the recovery of formononetin from preparative silica gel TLC was also determined by the comparison of the amounts of formononetin in the samples before and after separation by preparative TLC. The experiment for the recovery of formononetin during the preparative TLC was as follows: part of the extract (7.60 mg) was dissolved with MeOH (0.3 ml) and underwent preparative silica gel TLC (10 × 10 cm, 250 μm) for formononetin using the method described in the preparation of **sample 4**, and the relevant fraction was dissolved in MeOH (200 μl) and analyzed by HPLC-PDA.

Results

Based on our experiments, we determined the detection limit of formononetin (**1**) to be 60 ng/ml (0.6 ng) by HPLC-PDA. Based on the density of methanol (0.79 g/ml), this concentration is equal to 0.0759 ppm.

HPLC-PDA and LC-MS chromatograms of 80% MeOH/H₂O black cohosh extract (sample 1-a) revealed that there was no peak responsible for formononetin, suggesting that there was no formononetin in this black cohosh sample or the concentration of formononetin in the test sample was lower than 60 ng/ml. When a saturated methanolic solution of the 80% MeOH/H₂O

black cohosh extract (sample 1-b) was subjected to the analysis of formononetin by HPLC-PDA, four minor peaks were observed in the region between the retention times 45.0 and 46.5 min, where formononetin should appear, but none of them had a UV absorption spectrum similar to formononetin (Fig. 1). A comparison of the UV spectra of these four minor constituents with those of the major polyphenolics isolated from black cohosh indicated the similarity between the two groups of the UV spectra (Fig. 2), suggesting that instead of structures similar to isoflavonoids, the four minor constituents may possess structures similar to the derivatives of fukiic acid and piscidic acid, which were the major polyphenolics in black cohosh. This sample was further analyzed by LC-MS, and no signal was found in the TIC spectrum attributed to formononetin (Fig. 3A).

Since the solubility of formononetin in methanol is better than in 80% MeOH/H₂O, another sample was prepared for the analysis of formononetin by extracting black cohosh roots and rhizomes with methanol under reflux. The resulting extract was dissolved in methanol with the concentration about 50 mg/ml (sample 2). However, no peak responsible for formononetin in both HPLC-PDA and LC-MS chromatograms was observed. Although a peak was observed at the retention time close to formononetin on the LC-MS chromatogram with a SIM scan mode, this peak was finally determined not to be formononetin, via a spiking experiment (Fig. 3B and C).

When the black cohosh extract was prepared by extracting black cohosh roots and rhizomes which had been mixed with a small amount of formononetin (**1**), the peak of **1** in the HPLC-PDA chromatograms of the extract could be clearly observed. The peaks of formononetin (**1**) in samples 3-a and 3-b are shown in Fig. 4. The concentrations of **1** in samples 3-a and 3-b were 0.004407 and 0.0008011 mg/ml, respectively, and these two concentrations could be converted into the content of **1** in black cohosh roots and rhizomes as 0.01199% (119.9 ppm) and 0.0002398% (2.40 ppm).

A concentrated fraction for the analysis of formononetin was prepared following the published method (Panossian et al., 2004). Black cohosh plant material was extracted with methanol in an ultrasonic bath. The resulting black cohosh extract was further purified and concentrated for formononetin by preparative silica gel TLC, and the concentrated fraction (sample 4) was analyzed by HPLC-PDA and LC-MS, but formononetin was not detected. In the HPLC-PDA chromatogram, there were three minor peaks appearing in the range between the retention times 45 and 46 min. However, a comparison of the UV spectra of these three peaks with that of formononetin indicated a significant difference among the spectra, suggesting that none of them was formononetin. The fraction was further analyzed by

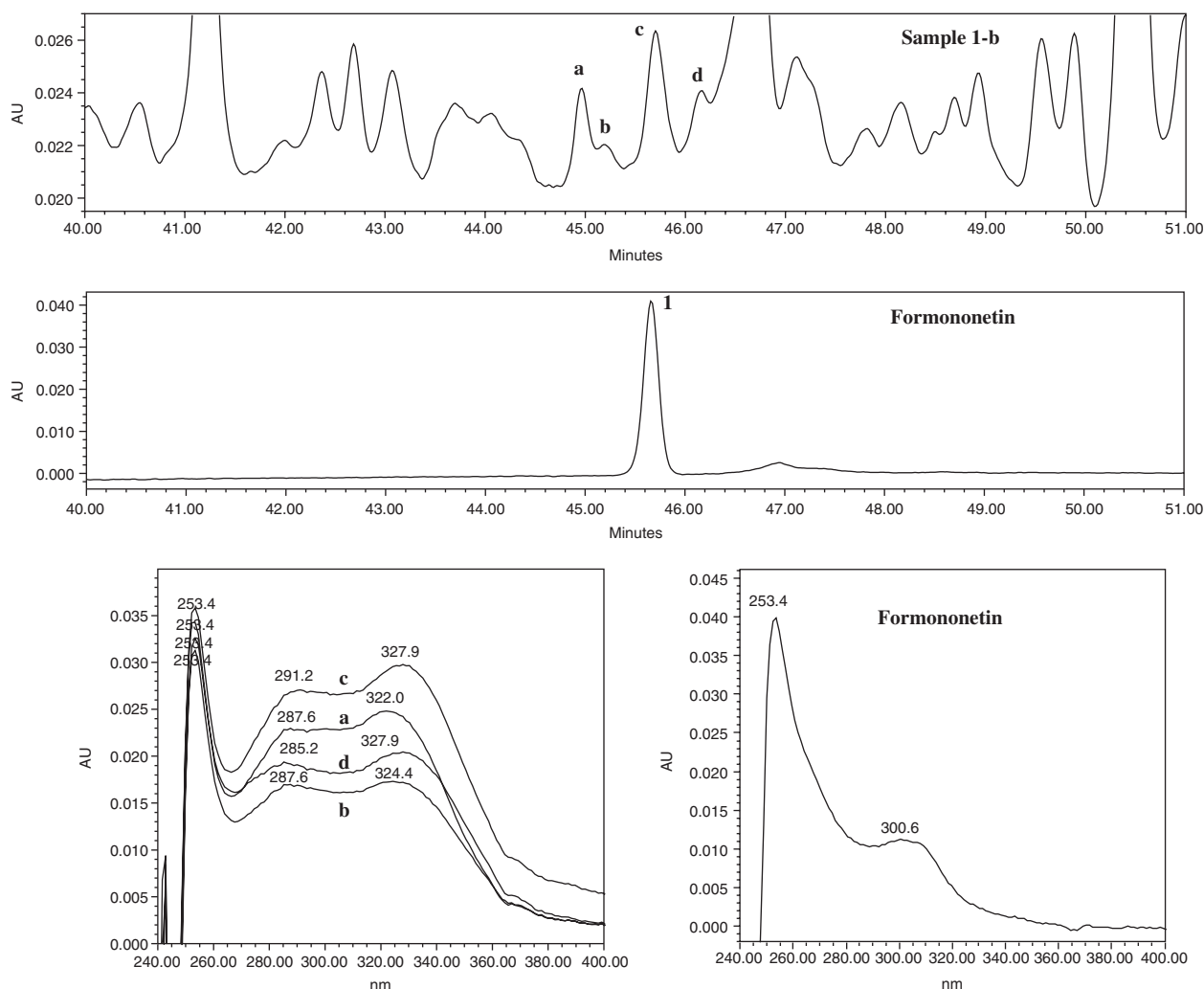


Fig. 1. HPLC-PDA chromatograms (258 nm) and UV spectra of formononetin and sample 1-b.

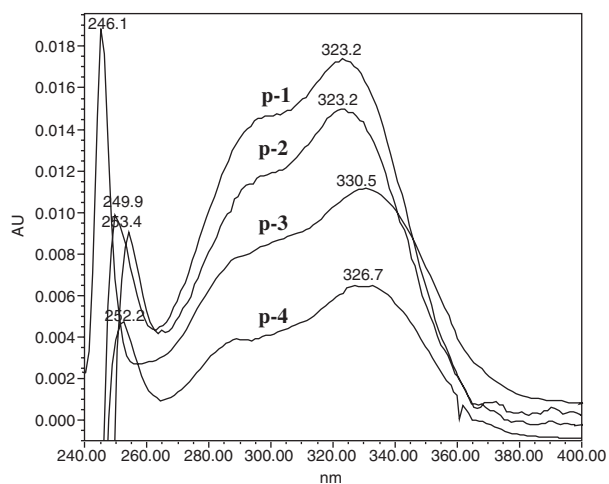


Fig. 2. UV spectra of four polyphenolics from black cohosh. **p-1**: Caffeic acid (2); **p-2**: ferulic acid (3); **p-3**: fukinolic acid (4); **p-4**: cimicifugic acid B (5).

LC-MS, and neither the full scan mode nor the SIM scan mode revealed any signal due to formononetin (Fig. 5B).

As a comparison, black cohosh extract which was prepared from the black cohosh roots and rhizomes spiked with a small amount of formononetin was also processed into a concentrated fraction (sample 3-c) for formononetin by preparative TLC. According to the HPLC-PDA and LC-MS chromatograms, the signal for formononetin was clearly observed in the spiked sample (Fig. 5C), suggesting that there was no detectable amount of formononetin in our test of black cohosh roots and rhizomes.

In this study, we also checked a black cohosh sample which had been stored for 86 years in The NYBG for formononetin. Our previous research on triterpene glycosides and phenolics of this 86-year-old black cohosh sample revealed that these two kinds of constituents were stable in this sample (Jiang et al.,

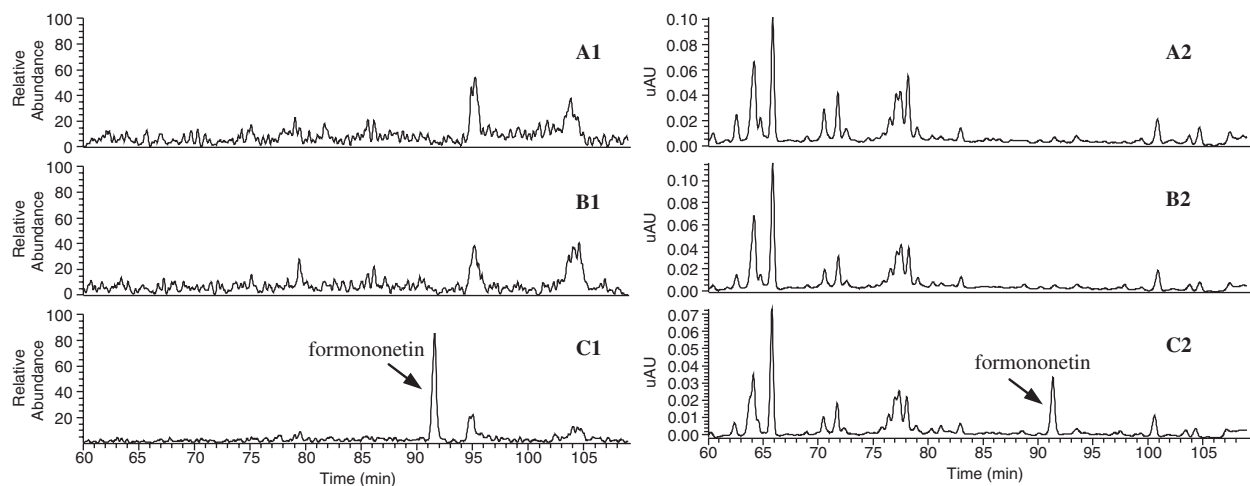


Fig. 3. LC-MS ESI-TIC chromatograms and UV spectra for samples 1-b and 2. **1:** SIM scan for the negative ion at 267 ± 0.5 ; **2:** UV spectra (258 nm). **A:** Sample 1-b; **B:** sample 2; **C:** sample 2 spiked with formononetin (**1**).

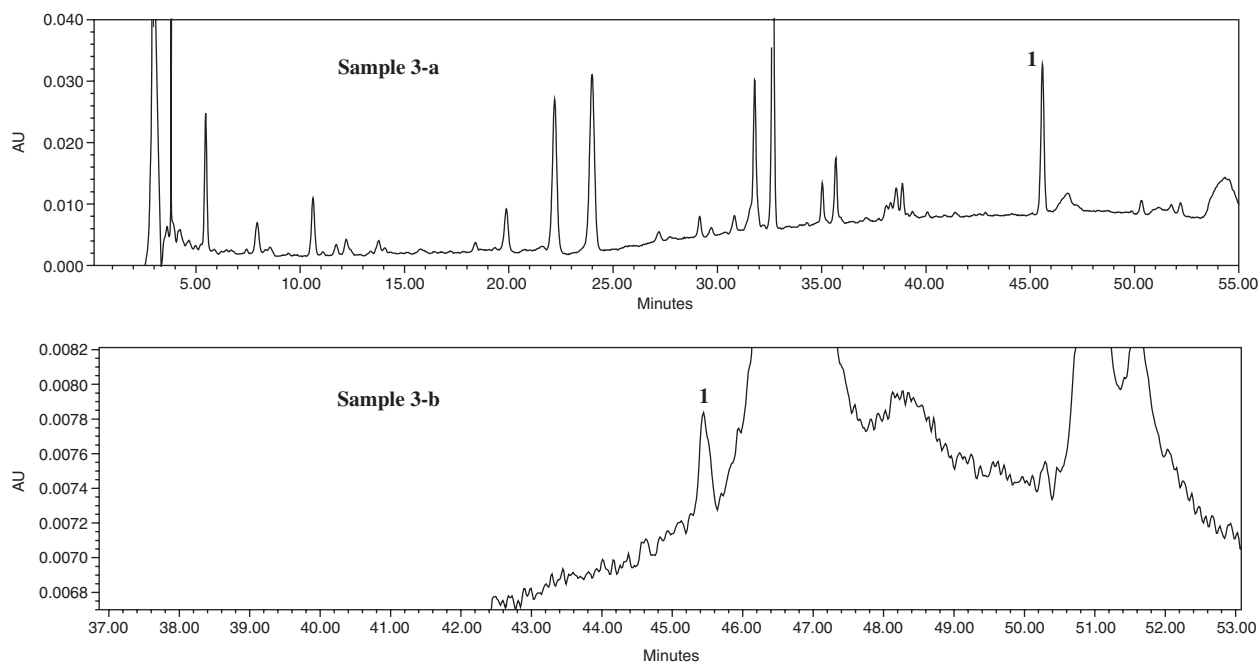


Fig. 4. HPLC-PDA chromatograms for formononetin (**1**) spiked in samples 3-a and 3-b (258 nm).

2005). The sample was processed using TLC to yield a concentrated fraction (sample 5) for the HPLC-PDA analysis of formononetin. However, there was no evidence to show the presence of formononetin in the 86-year-old black cohosh (Fig. 6).

The recovery of formononetin for the extraction of black cohosh roots and rhizomes with 80% MeOH/H₂O was determined to be 98.56–103.37% by the HPLC-PDA method. The recovery of formononetin for concentrating the fraction by silica gel TLC was determined to be 90.22–94.40%.

Discussion

For most polyphenolic compounds including flavonoids and isoflavonoids, the HPLC-PDA method is a reliable and sensitive analytical method due to the strong UV absorption of these polyphenols (Hasler et al., 1992; Ito et al., 1988; Sagara et al., 1987; Wang and Chen, 1999). As an isoflavone, formononetin (**1**) possesses strong UV absorption and is easily detected by UV at 248.7 nm. Therefore, it was not reasonable for Panossian et al. to suggest the HPLC-PDA analysis of

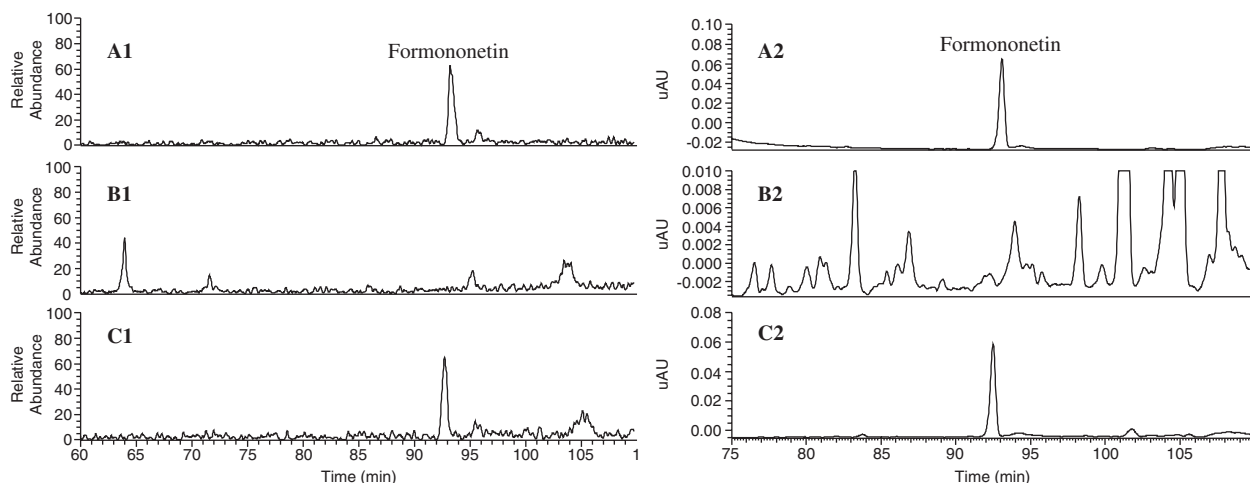


Fig. 5. LC-MS chromatograms and UV spectra of formononetin, sample 4, and sample 3-c. 1: ESI-TIC of SIM scan for the negative ion at 267 ± 0.5 ; 2: UV spectra (258 nm). A: Formononetin; B: sample 4; C: sample 3-c.

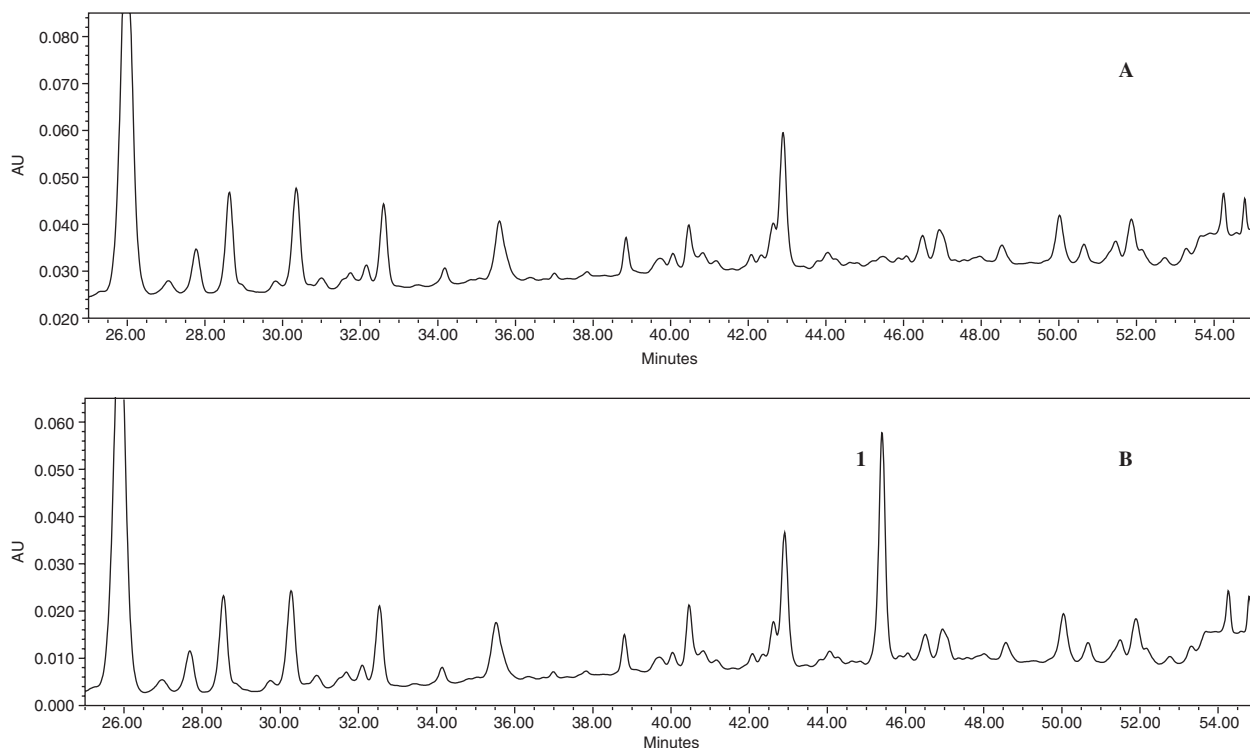


Fig. 6. HPLC-PDA chromatograms for the 86-year-old black cohosh sample (258 nm). A: Sample; B: sample spiked with formononetin (1).

formononetin is an incorrect method (Panossian et al., 2004). In fact, according to our research, the sensitivity of the HPLC-PDA method for the analysis of formononetin was similar to that of the reported fluorescent method. The limits of detection of the HPLC-PDA method and the reported fluorescent method were 0.0759 ppm (0.00000759%) and 0.08 ppm (0.000008%), respectively (Panossian et al., 2004). Based

on the recovery of formononetin for concentrating the fraction by silica gel TLC, the concentration of formononetin in sample 4 for the HPLC-PDA and LC-MS analysis should be 0.04049 mg/ml, which was much higher than the limited detectable concentration of formononetin (60 ng/ml) by the HPLC-PDA and LC-MS methods. It is not possible that formononetin in such high concentration could not be detected by

HPLC-PDA and LC-MS method. Moreover, when a more efficient extraction method was used to extract black cohosh plant material (sample 2), formononetin still could not be found in the extract by HPLC-PDA and LC-MS analysis even though a very concentrated solution was used for the analysis. Therefore, analytical results from our present study confirmed our previous report that there was no detectable amount of formononetin in black cohosh roots and rhizomes.

According to the research from our group and published reports, black cohosh contains many phenolic constituents, including caffeic acid, fukinolic acid and cimicifugic acid B, which displayed similar UV absorption spectra (Fig. 2), but the retention times of these phenolic constituents differed significantly (Jiang et al., 2005; Li et al., 2003). For example, the retention times of caffeic acid and cimicifugic acid B are about 12 and 33 min, respectively, in our experiments. Results from another research group also indicated that caffeic acid derivatives and ferulic acid derivatives could elute in a range of retention times from 4 to 40 min when they were analyzed by HPLC, suggesting that these types of phenolic constituents can possess varied polarities. Thus for sample 1-b, it is reasonable to deduce that the four minor constituents that elute in the region between retention times 45.0 and 46.5 min (Fig. 1) are the derivatives of fukiic acid and piscidic acid due to the similarity of the UV spectra.

In a separate experiment, we analyzed ten commercially available black cohosh products for polyphenolic constituents by HPLC-PDA and LC-MS methods, and once again we did not detect any trace of formononetin in the products. Furthermore, studies from other research groups also showed that no detectable formononetin was found in a variety of black cohosh samples using HPLC-PDA, IR, and NMR methods (Li et al., 2002; McCoy and Kelly, 1996; Struck et al., 1997).

In our previous research, we analyzed 13 populations of black cohosh which were collected from different localities in the eastern United States, covering the areas from New York to North Carolina. In this study, we checked two black cohosh samples which were collected in different times with an intervening span of 86 years. However, none of these black cohosh samples were found to contain any trace of formononetin. This suggested that black cohosh distributed in America may not contain this isoflavone at all.

Several reasons are conceivable to explain the controversial results of Panossian et al. regarding the presence of formononetin in black cohosh. One possibility is that the method they described is not specific enough for the analysis of formononetin in black cohosh. According to our study, when the black cohosh extract was developed on silica gel TLC using the reported mobile phase (Panossian et al., 2004), there were many compounds with the same retention time as

formononetin. Some compounds had UV absorption at 254 nm similar to formononetin. Therefore, it is reasonable to deduce that some compounds, which had an R_f value similar to formononetin in TLC, may possess the fluorescent property similar to formononetin in black cohosh as well. This deduction can be simply verified by Panossian's own research. In Fig. 5 of Panossian's paper (the fluorescent TLC-densitometry scans of formononetin), there was more than one peak observed in the black cohosh sample range from 20.0 to 40 mm, where formononetin should appear (Panossian et al., 2004). Therefore, it does not appear to be reliable to identify formononetin in black cohosh based on the fluorescent property and R_f value only. Moreover, since the separation of compounds by TLC is often not as efficient as by HPLC, care must be exercised to conclude a quantitative analysis based on TLC, due to constituents sharing the same R_f .

Another possibility was that the plant material Panossian et al. used was different from ours. There have been many papers reporting that when plants of the same species are collected from different regions (Hao et al., 1997) or during different harvesting seasons, the plants reveal different chemical profiles (Wang and Chen, 1999). Unfortunately, Jarry et al. did not state the place of collection for the black cohosh they studied. Meanwhile, Panossian et al. only described that the black cohosh they used was purchased from a German company (Jarry et al., 1985; Panossian et al., 2004). Considering that both Jarry and Panossian's research groups were in Europe and the black cohosh plant material may be collected in Europe, the possibility exists that the chemical constituents of black cohosh plant materials collected from America and Europe differ from one another. Black cohosh distributed in Europe may contain formononetin while the same plant grown in America may not possess this compound. Therefore, it may be useful to investigate and compare the chemical constituents of black cohosh plant materials harvested from America and Europe for better understanding the similarity and difference of the chemical profiles of the black cohosh from different continents.

As one of the largest classes of plant phenolics, flavonoids are found in almost all plant families including the family Ranunculaceae (Bylka, 2002; Egger and Keil, 1965; Lebreton, 1986; Tofeni and Popescu, 1997). Therefore, it would not be unusual if some flavonoids were obtained from black cohosh. However, unlike flavonoids, isoflavonoids are only reported from species in several families. For example, formononetin has been detected in a number of species in the Fabaceae (Bell and Charlwood, 1981; Taiz and Zeiger, 1991), and there have been a few reports of its occurrence in other plant families, including the Myristicaceae (Braz Fo et al., 1977; Lopes et al., 1999) and Iridaceae (Wang and

Chen, 1999; Xu et al., 1999). Therefore, isoflavonoids like formononetin have been used for chemotaxonomical markers (Nigel et al., 2003). We have not found any reports about the isolation of isoflavonoids from species of the genus *Actaea* or the family Ranunculaceae except for the previously cited reports of formononetin from black cohosh (Jarry et al., 1985; Panossian et al., 2004). Jarry et al. also remarked that the isolation of formononetin from *Cimicifuga racemosa* (now considered *Actaea racemosa*) was unexpected and unusual (Jarry et al., 1985). Plants in the same genus often have similar secondary metabolites. It is unexpected that, among hundreds of species of Ranunculaceae that have been studied, an isoflavone has only been reported from black cohosh. Perhaps it is also possible that the black cohosh could be contaminated with an isoflavonoid-producing plant or contaminated by some unknown factor during the experiments.

According to previous researchers, the concentration of formononetin necessary to stimulate in vitro the transcriptional activity of the estrogenic receptor is 10^{-6} M (268 ng/ml) (Miksicek, 1994). Therefore, even if black cohosh and the products did contain formononetin but its amount was lower than the limit of detection of the HPLC-PDA method (60 ng/ml), the concentration of formononetin in black cohosh extract would be much lower than that necessary to stimulate the estrogenic receptor based on the solubility of black cohosh extract. Therefore, it is also not reasonable to attribute the estrogen-like activity of black cohosh extracts to formononetin.

HPLC-PDA and LC-MS analyses are reliable methods for detection of formononetin, with the limits of detection lower than 0.08 ppm (0.000008%). The analytical results from our present study using HPLC-PDA and LC-MS indicated that there is no detectable amount of formononetin in any test black cohosh samples including black cohosh roots and rhizomes and black cohosh products.

Acknowledgements

We thank Drs. Koji Nakanishi, Yoshihiro Itagaki, and Young Pyo Jang for their helpful suggestions. We are grateful to the Edith C. Blum Foundation for their support at this project. This project was made possible by Grant # P50-AT00090 from the National Institutes of Health-National Center for Complementary and Alternative Medicine (NIH-NCCAM). Its contents are solely our responsibility and do not necessarily reflect the official views of NIH-NCCAM. The authors are thankful to PureWorld Botanicals Inc. (South Hackensack, NJ) for providing black cohosh for this study.

References

- Barton, B.S., 1798. Collections for an Essay Towards a Material Medica of the United States. Way & Groff, Philadelphia.
- Bell, E.A., Charlwood, B.V., 1981. Secondary Plant Products. Springer, Berlin, Heidelberg, New York.
- Borrelli, F., Izzo, A.A., Ernst, E., 2003. Pharmacological effects of *Cimicifuga racemosa*. Life Sci. 73, 1215–1229.
- Braz Fo, R., Gottlieb, O.R., De Moraes, A.A., Pedreira, G., Pinho, S.L.V., Magalhaes, M.T., De S Ribeiro, M.N., 1977. The chemistry of Brazilian Myristicaceae. IX. Isoflavonoids from Amazonian species. Lloydia 40, 236.
- Bylka, W., 2002. Flavonoids in the leaves with stems of some species of the *Aquilegia* L. genus. Acta Pol. Pharmaceut. 59, 57–60.
- Egger, K., Keil, M., 1965. Flavonol glycosides in Ranunculaceae, distribution of the glycoside types. Ber. Deut. Bot. Ges. 78, 418–422.
- Hao, X., Nie, J., Sun, H., Gu, Z., 1997. The chemotaxonomy of *Spiraea japonica* complex. Acta Bot. Yunnanica 19, 297–303.
- Hasler, A., Sticher, O., Meier, B., 1992. Identification and determination of the flavonoids from *Ginkgo biloba* by high-performance liquid chromatography. J. Chromatogr. 605, 41–48.
- Ito, Y., Hirayama, F., Suto, K., Oshima, T., Sagara, K., Yoshida, T., 1988. Determination of flavonol glycosides in *Epimedium herba* by high-performance liquid chromatography. J. Chromatogr. 456, 392–397.
- Jarry, H., Harnischfeger, G., Düker, E.M., 1985. Studies on the endocrine efficacy of the constituents of *Cimicifuga racemosa*: 2. In vitro binding of constituents to estrogen receptors. Planta Med. 51, 316–319.
- Jiang, B., Yang, H., Nuntanakorn, P., Balick, M.J., Kronenberg, F., Kennelly, E.J., 2005. The value of plant collections in ethnopharmacology: a case study of an 85-year-old black cohosh (*Actaea racemosa* L.) sample. J. Ethnopharmacol. 96, 521–528.
- Kennelly, E.J., Baggett, S., Nuntanakorn, P., Ososki, A.L., Mori, S.A., Duke, J., Coleton, M., Kronenberg, F., 2002. Analysis of thirteen populations of black cohosh for formononetin. Phytomedicine 9, 461–467.
- Lebreton, P., 1986. Flavonoids, taxonomic markers in the Ranunculaceae. Plant. Med. Phytother. 20, 275–286.
- Li, W., Chen, S., Fabricant, D., Angerhofer, C.K., Fong, H.H.S., Farnsworth, N.R., Fitzloff, J.F., 2002. High-performance liquid chromatographic analysis of black cohosh (*Cimicifuga racemosa*) constituents with in-line evaporative light scattering and photodiode array detection. Anal. Chim. Acta 471, 61–75.
- Li, W.K., Sun, Y.K., Liang, W.Z., Fitzloff, J.F., van Breemen, R.B., 2003. Identification of caffeic acid derivatives in *Actaea racemosa* (*Cimicifuga racemosa*, black cohosh) by liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 17, 978–982.

- Lopes, N.P., Kato, M.J., Yoshida, M., 1999. Antifungal constituents from roots of *Viola surinamensis*. *Phytochemistry* 51, 29–33.
- McCoy, J., Kelly, W., 1996. Survey of *Cimicifuga racemosa* for phytoestrogenic flavonoids. Book of abstract, 212th ACS National Meeting, pp. 25–29.
- Miksicek, R.J., 1994. Interaction of naturally occurring nonsteroidal estrogens with expressed recombinant human estrogen receptor. *J. Steroid Biochem. Mol. Biol.* 49, 153–160.
- Nigel, C.V., Sutton, P.S.E., Kite, G.C., Ireland, H.E., 2003. Six new isoflavones and a 5-deoxyflavonol glycoside from the leaves of *Ateleia herbert-smithii*. *J. Nat. Prod.* 66, 210–216.
- Panossian, A., Danielyan, A., Mamikonyan, G., Wikman, G., 2004. Methods of phytochemical standardisation of rhizoma *Cimicifugae racemosae*. *Phytochem. Anal.* 15, 100–108.
- Rafinesque, C.S., 1828. *Medical Flora or Manual of the Medical Botany of the United States of North America*. Atkinson & Alexander, Philadelphia.
- Sagara, K., Oshima, T., Sakamoto, S., Yoshida, T., 1987. Determination of coumarins in *Cnidium monnieri* fructus by high-performance liquid chromatography. *J. Chromatogr.* 388, 448–454.
- Struck, D., Tegtmeier, M., Harnischfeger, G., 1997. Flavones in extracts of *Cimicifuga racemosa*. *Planta Med.* 63, 289.
- Taiz, L., Zeiger, E., 1991. *Plant Physiology*. The Benjamin/Cummings Publishing Company, Inc., Redwood.
- Tofeni, A., Popescu, H., 1997. Flavonoids from *Adonis aestivalis* L. (Ranunculaceae). *Clujul Med.* 70, 287–291.
- Wang, M.Z., Chen, Y.H., 1999. *HPLC Analyses of Traditional Chinese Medicinal Herbs Most in Use*. Science Press, Beijing.
- Xu, Y.L., Ma, Y.B., Xiong, J., 1999. Isoflavonoids of *Iris tectorum*. *Acta Bot. Yunnanica* 21, 125–130.