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Hussein Al-Amier
Al-Azhar University, Nasr City, Egypt, halamier66@hotmail.com

Stephen J. Eyles
University of Massachusetts, eyles@polysci.umass.edu

Lyle Craker
University of Massachusetts, craker@umass.edu

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Evaluation of Extraction Methods for Isolation and Detection of Formononetin in Black Cohosh (*Actaea racemosa* L.)

Hussein Al-Amier[1,2*], Stephen J. Eyles[3], and Lyle Craker[1]

[1]Medicinal Plant Program, University of Massachusetts, Amherst, MA 01003, U.S.A.
[2]Horticulture Department, Faculty of Agriculture, Al-Azhar University, Nasr City, Cairo, Egypt
[3]Polymer Science and Engineering, University of Massachusetts, Amherst, MA 01003, U.S.A.

***Corresponding author:** halamier66@hotmail.com

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Abstract

Black cohosh (*Actaea racemosa* L.) is a medicinal plant from which extracts of the roots and rhizomes (commonly known as rhizomes) have become a popular remedy for the relief of menopausal symptoms. While the chemical constituents responsible for the medicinal properties of the plant are unknown, extracts of the rhizomes are known to contain cycloartane triterpene glycosides, phenolics, and flavonoids. The possible presence of the phytoestrogenic isoflavone formononetin (7-hydroxy-4'-metoxy-isoflavone), however, could indicate that using black cohosh by women could enhance the risk of breast cancer similar to the risks associated with estrogen. Analyses for formononetin in black cohosh over the past several years, however, has been conflicting with some laboratories reporting the constituent in the rhizomes and others not. To determine whether these differences could be due to differences in the extraction and analysis methodology, extractions of the rhizomes were made with methanol, ethanol, and isopropanol and purified using a SPE C18 and DEAE Sephadex A-25 column. LC-MS-MS was used to detect the presence of formononetin using a Waters 2690 Alliance HPLC coupled with a Micromass Quattro 11 triple-quadruple mass spectrometer equipped with an electrospray source operated in the positive/negative ionization mode. The mass spectra of black cohosh constituents were obtained by infusing standard solutions into the mass spectrometer or by LC-MS, LC-MS-MS with product ion scanning. Formononetin at a concentration of <125 ng g^{-1} was identified in methanol extracts using LC-MS-MS with a microbore C18 column and with the ion pairs of *m/z* 269-253, 269-225, and 269-197.

Introduction

In the United States, some 25 to 40 percent of menopausal women have traditionally used some form of hormone replacement therapy (HRT) during or following menopause (Handa et al., 1996; Keating, et al., 1999; Stehman-Breen, et al., 1999). Reports during the past few years of adverse effects from HRT (Bush and Whitemen, 1999; Grodstein, et al., 1996), such as increased risk of breast and uterine cancer, have caused nationwide concern (Beckner, 2002). As result of the warnings about HRT, many women have been using botanical dietary supplements as an alternative to HRT with one of the more popular of these supplements being the roots and rhizomes (commonly referred to as rhizomes) of black cohosh (*Actaea racemosa* L.), a perennial plant native to the eastern woodlands of North America that was used by Native Americans as a therapeutic agent (Wood, 2000).

Formononetin, an isoflavone, was initially proposed to be responsible for the pharmacological activity of black cohosh due to the potential phytoestrogenic activity of the compound (Kennely, et al., 2002). Such estrogenic activity, however, could

possibly stimulate malignances similar to those instigated by HRT, making the presence of formononetin in the black cohosh rhizome extracts a potential danger to consumers. Formononetin was first reported to be in black cohosh rhizomes by Jarry, et al. (1985) after analysis of a methanol extract by TLC, HPLC-UV, IR, MS and NMR. Later, Li, et al. (2003) used positive ion electrospray mass spectra to detect caffeic acid derivatives in a methanol extract of a commercial source of black cohosh rhizomes by comparing retention times of the plant tissue extracts with standards and the *M/Z* values in MRM and the MRM absolute response ratios during LC-MS-MS (detection limit 10 pg), but detected no formononetin. Panossian, et al. (2004) reported the use of TLC-fluorometry to detect from 3.1 to 3.5 μg formononetin per g dry tissue in the methanol extract and methanolysis products of rhizomes and roots of black cohosh. Freeburg, et al. (2007), using HPLC and GC-MS, reported that in an analysis of fresh black cohosh rhizomes, a reasonable profile match to formononetin was detected in a mature rhizome sample, but not in the roots, young rhizomes, nor lyophilized samples.

More recent studies, however, have failed to detect the compound in crude plant material or extracts. Struck, et al. (1997), using HPLC, reported that the isoflavone formononetin and the flavonol kaempferol were not detectable in an ethanol extract (60% v/v) or isopropanol extract (40% v/v) of black cohosh rhizomes, suggesting that either the compounds were below the detection limit (1 μg mL^{-1}) or not present in the extract. Li, et al. (2002) used HPLC coupled with a photodiode array detector (PDA) and an evaporative light scattering detector (ELSD) in an attempt to detect formononetin and kaempferol in a methanolic extract of black cohosh, but had no success with detection limits in the range of 26-55 ng per 10 μL injection. Kennelly, et al. (2002) analyzed commercial black cohosh products and rhizomes collected from wild populations of the plant using an 80 percent methanol extract that was purified by solid phase extract (SPE) and analyzed by HPLC-PDA and LC-MS, but no formononetin was detectable in any of the samples. Jiang, et al. (2006a) evaluated commercial rhizomes samples and powdered encapsulated products using 80 percent methanol, 75 percent ethanol, and 40 percent 2-propanolic extracts for the presence of formononetin using HPLC-PDA and LC-SIM-MS, but no formononetin was detectable. In a second study, Jiang, et al. (2006b) used TLC-PDA and LC-MS to analyze methanol and aqueous methanol extracts of black cohosh roots and rhizomes of commercial samples and an 86 year old herbarium specimen, but could detect no formononetin.

Differences within and among laboratories for the presence of formononetin in black cohosh have left questions as to whether the compound is present in the plant tissue. In the present study, selected solvent systems were evaluated to optimize extraction of any formononetin and to conclusively determine whether the compound was a constituent of black cohosh.

Materials and Methods

Plant material. Black cohosh (*Actaea racemosa* L. [syn. *Cimicifuga racemosa* (L.) Nutt.] rhizomes, originating from plant material collected from 13 locations in the eastern United States (Figure 1), were used in this study. After collection, the original rhizomes were cultivated in outdoor beds for one growing season in Amherst, Massachusetts, and, after these cultivated plants had produced mature seeds in late summer, the underground roots and rhizomes (commonly known as rhizomes) of 32 individual plants were harvested. The harvested rhizomes were washed thoroughly with water to remove extraneous matter and then dried in a mechanical dryer at 55 ^{o}C for 48 h. The dried rhizomes from all the cultivated plants were collectively mixed together for a primary bulk sample. This bulk sample was frozen in liquid nitrogen and then ground to a fine powder with a mortar and pestle.

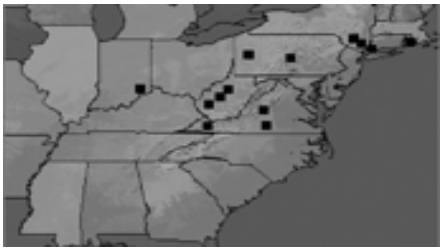

Figure 1. Originating sources of black cohosh samples. Black boxes indicate sample site.

Experimental. To determine if the extraction procedure or solvent affected detection of formononetin in the black cohosh tissue, the powdered primary bulk sample (90 g) was divided into nine subsamples of 10 g each. Following a version of the extraction method used by Li, et al. (2002a), the weighed subsamples were placed in separate 250 mL round-bottom flasks and 50 mL of one of the following solvents was added to each flask: 50, 75, or 100 percent methanol; 50, 75, or 95 percent ethanol; or 50, 75, or 100 percent isopropanol for extraction of any formononetin. In brief, individual samples were mixed with the solvent by agitating with a magnetic stirrer for 48 h. The agitated samples were then sonified for 30 min at 30 to 40 ^{o}C, using a Misonix S-4000 sonicator. The sonified samples were subsequently cooled to room temperature and filtered through Whatman No. 1 filter paper into clean 500 mL round bottom flasks. The residue for each sample was returned to the original 250 mL flasks and a second 50 mL of the solvent was added and the agitation and sonication were repeated. This extraction procedure was done on each sample a total of four times with the extracts from each sample combined. Each of the combined extracts from the nine subsamples was divided into three portions, making a total of 27 samples for analysis. Throughout the extraction and analyses, all glassware was wrapped with aluminum foil to prevent any light induced degradation of formononetin.

The first portion of each solvent extract was evaporated to dryness, under vacuum, at 45 to 50 ^{o}C. The resulting residue (a crude extract) was redissolved by washing the flask two times with 4 mL of the high concentration of the original solvent (100 % methanol, 95 % ethanol or 100 % isopropanol), filtered through Whatman No. 1 filter paper to remove any solid particles and then transferred to a 10 mL volumetric flask and made to volume with the solvent used for washing.

The second portion of each solvent extract was evaporated to dryness, redissolved in 4 mL of methanol, and purified by solid phase extraction using a C18 column (Agilent, Santa Clara, CA) (1 cm in diameter x 5 cm in length) that had been preconditioned by successive 5 mL additions of hexane, acetonitrile, and 5% methanol containing 0.5% acetic acid. The preconditioned column was washed three times with distilled, deionized water and the sample was applied and eluted by addition of 5 mL of HPLC grade methanol at a flow rate of 0.2 mL min^{-1}. The eluted sample was brought to volume in 10 mL of methanol.

The third portion of each solvent extract was evaporated to dryness and then re-dissolved in 30 mL of a chloroform:methanol:water (8:4:3; v:v:v) mixture contained in separatory funnel. The lower phase chloroform layer was separated and dried. The dried residue was re-dissolved in 0.5 mL methanol and subjected to anion exchange purification using a DEAE Sephadex column (1 cm in diameter x 5 cm in length) that had been converted to the hydroxyl form by successive washing with 0.1 M sodium hydroxide in 70% methanol, 70% methanol, and methanol (Mazur et al., 1996). The converted DEAE column was repacked with HPLC grade methanol and used immediately after conversion. The sample was added to the column, eluted with 5 mL of HPLC methanol containing 0.1 M acetic acid, evaporated to dryness, and redissolved in 1 mL of HPLC grade methanol and brought to volume in 10 mL of methanol.

Analysis. For analysis, all the prepared black cohosh extracts were filtered through a 0.22 µm MCE membrane (Millipore syringe filter) to remove any debris and then subjected to LC-MS-MS for detection of formononetin (Figure 2). Samples of the extracts were separated by nano-HPLC using a Dionex Ultimate nanoLC system equipped with a PepMap100 C18 (75 µm x 5 cm) column. Sample injections (1 µL) were eluted by a 5-95% gradient (H_2O, 0.1% formic acid:acetonitrile, 0.1% formic acid) over 60 min with a flow rate of 150 nL min^{-1}. The eluent was interfaced to a QStarXL hybrid quadrupole time of flight mass spectrometer (MDS/Sciex, Applied Biosystems, Framingham, MA) using continuous nanoflow source. Acquisition was set to measure interlaced full scan MS and product ion of m/z 269 in positive ion mode. Extracted ion chromatograms (multiple reaction monitoring) were reconstructed for the ion pairs m/z 269->253, 269->225 and 269->197, characteristic of formononetin (Figure 3).

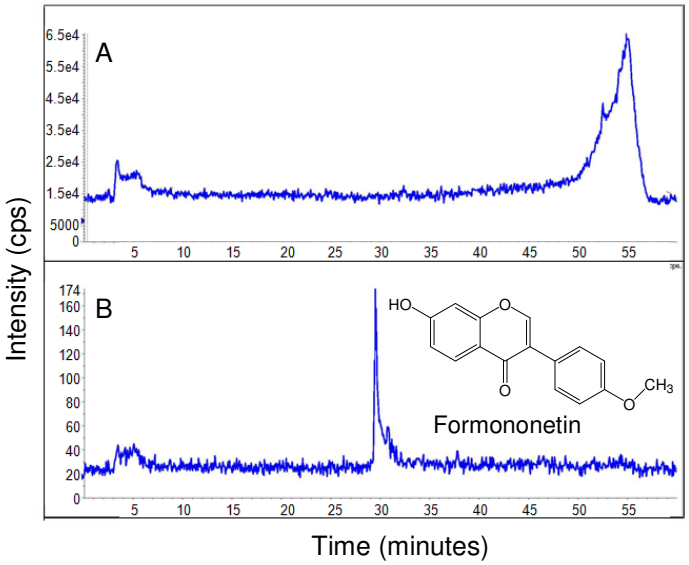

Figure 2. LC-MS and LCMS/MS chromatograms of formononetin standard.
Intensities were measured in arbitrary units.
A = Total ion chromatogram full scan TOF.
B = Total ion chromatogram for product ion scan (parent ion m/z 269);
Formononetin elutes at approximately 30 min.

Results

Formononetin was detected in the mixed sample of black cohosh roots and rhizomes, but this finding was dependent upon the solvent system and extraction methodology (Figure 4, Table 1). Only the crude extract of samples sonicated in methanol contained any detectable formononetin. Within the solvent range tested (50-100%), the methanol concentrations of the extracting solution were equally effective in extracting the formononetin. No formononetin was detected in samples subjected to solid phase extraction or DEAE.

Discussion

The type and level of secondary metabolites observed in plants can vary significantly, depending upon the plant genome, the environmental conditions in which the plant is growing, the stage of plant development, and post-harvest handling (Arias-Castro, et al, 1993). Indeed, a constituent may be present only under certain environments (McMurray, et al. 1986; Arias-Castro, et al., 1993; Sivesind and Seguin, 2005), or plant development stage (Arias-Castro, et al, 1993; Freeburg, et al., 2007). Detection of some plant constituents is difficult due to low concentrations, extraction procedures, and source purification (Freeburg, et al., 2007).

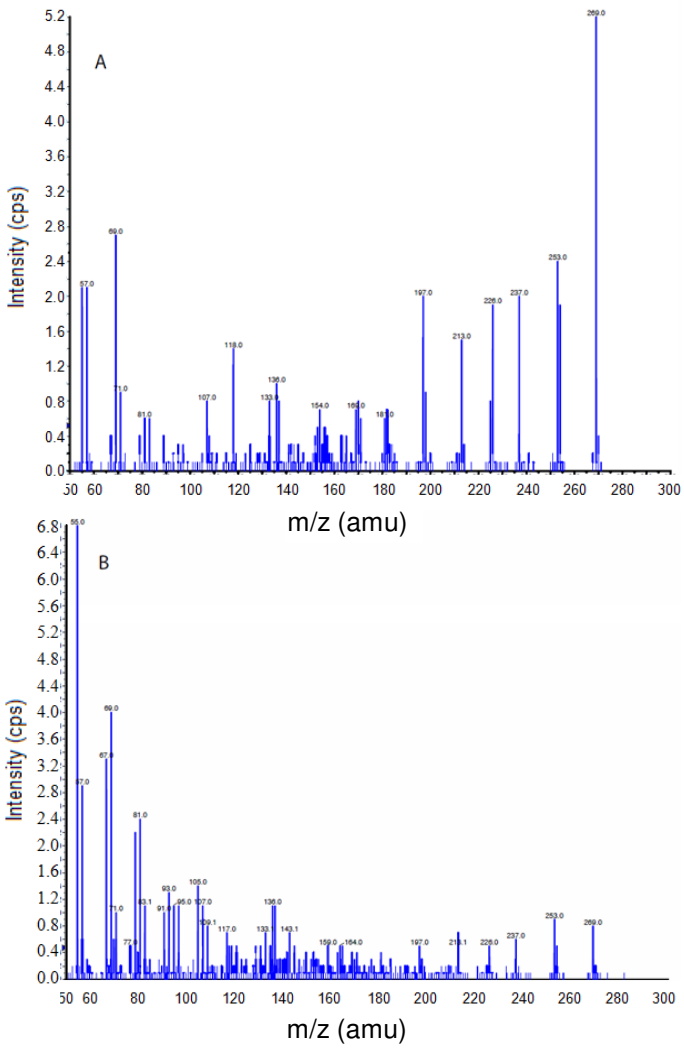

Figure 3. MS/MS product ion mass spectra from parent ion m/z 269.
Intensities were measured in arbitrary units.
A = Formononetin standard
B = Black cohosh 100% methanol extract chromatograph peak, 29.5-30.2 min elution.

The prevailing techniques for isolation of flavones from plant sample include simple shaking, Soxhlet extraction, and ultrasonification with organic solvents. Any extraction or analysis of isoflavones in biological samples is, however, complicated due to time-consuming sample preparation and low efficiency of chromatographic separation. A com-

bination of highly effective and selective isolation and purification procedures with effective and sensitive separation methods is necessary for the quantification and identification of compounds present at low levels in biological samples. For isoflavones, the commonly used methodology has been high-performance liquid chromatography (HPLC) coupled with mass spectrometric (MS) detection (Li, et al., 2002).

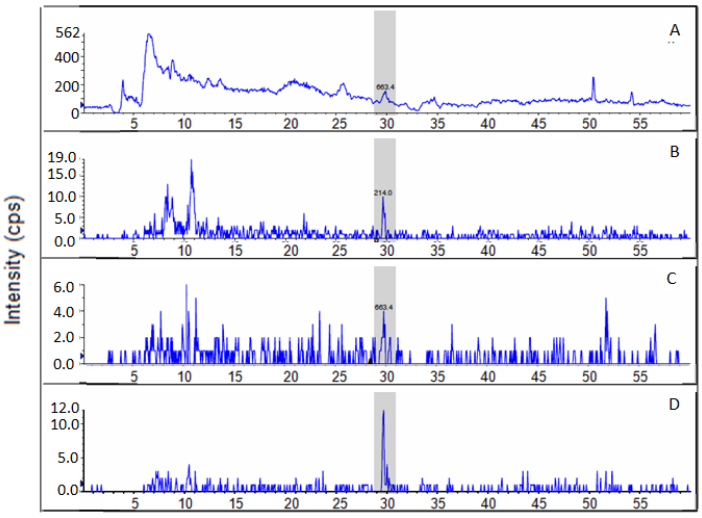

Figure 4. LC-MS/MS obtained from 100% methanol extract. Intensities were measured in arbitrary units.
A = Total ion chromatogram
B-D = Product ion scans of parent m/z 269 at m/z 197, m/z 253. And m/z 253.

Table 1. Effect of solvent and extraction method on formononetin detection in black cohosh.

Solvent	Concentration (%)	Extraction method[1]		
		Crude	SPE	DEAE
Methanol	50	+	-	-
	75	+	-	-
	100	+	-	-
Ethanol	50	-	-	-
	75	-	-	-
	95	-	-	-
Isopropanol	50	-	-	-
	75	-	-	-
	100	-	-	-

[1] + = formononetin detected, - = formononetin not detected.

In our study, the choice of the solvent and sample preparation system impacted the constituent profile obtained from the black cohosh rhizome samples, with formononetin detectable only in the methanol extract. Low formononetin levels and limited recovery of extracts purified by SPE C18 or DEAE apparently prevented detection of formononetin. Our study supported the concept of extraction selectivity being correlated with the diluent parameter (DP) of the organic solvent (Walkowiak, *et al*, 1992) and being dependent on solvent polarity Lin and Giusti (2005) with isoflavones. Only the methanol extraction had a detectable amount of the isoflavone formononetin.

Our results differ from the finding of other investigators (Struck, et al., 1997; Kennelly et al., 2002) that concluded formononetin does not exist in black cohosh rhizomes. The differences in results may be due to differences in selection and location of plant material (McMurray, et al., 1986; Arias-Castro, et al.,1993; Sivesind and Seguin, 2005), extraction protocols, and/or instrumental detection limits for formononetin used in their studies. The level of formononetin in the rhizome samples were quite low (<125 ng g^{-1}), making them undetectable by conventional high performance liquid chromatography. Earlier studies (Jarry and Harnischfeger, 1985; Jarry et al., 1985) mentioned the presence of formononetin as a constituent of black cohosh, but could not be verified. This study verified the presence of formononetin in black cohosh rhizomes and developed a relatively fast, reliable, sensitive, and precise method for extraction, purification, and detection of the isoflavone formononetin.

Acknowledgements

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