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Supercritical Fluid Extraction and High-Performance Liquid Chromatographic Determination of Phloroglucinols in St. John's Wort (*Hypericum perforatum* L.)

YANYAN CUI AND CATHARINA Y. W. ANG*

Division of Chemistry, National Center for Toxicological Research, U.S. Food and Drug Administration, HFT-230, 3900 NCTR Road, Jefferson, Arkansas 72079

A small-scale supercritical fluid extraction (SFE) method was developed for the selective extraction of phloroglucinols from St. John's wort (SJW) leaf/flower mixtures using supercritical carbon dioxide (CO_2). The extraction efficiency was investigated as influenced by pressure, temperature, time, and modifier. The optimized condition of SFE was carried out at 3.80×10^4 kpa (5500 psi) and 50 °C. Samples were held in static extraction for 10 min, followed by a dynamic extraction for 90 min at the flow rate of 1 mL/min. A simple and sensitive HPLC method was developed for the analysis of hyperforin and adhyperforin, the major phloroglucinols, in the SFE extract of SJW.

KEYWORDS: ***Hypericum perforatum* L.; St. John's wort; supercritical fluid extraction; hyperforin; adhyperforin**

INTRODUCTION

St. John's wort (*Hypericum perforatum* L.) (SJW) is a widely distributed herbaceous perennial plant, recently well-known as a medicinal plant in Europe and the U.S. for the treatment of mild to moderate depression. The efficacy and safety aspects of SJW have been demonstrated in many clinical trials, and the use of this herb has challenged conventional antidepressant drugs (*1*, *2*). More than 10 components have been found in SJW, including flavonoids (rutin, hyperoside, isoquercitrin, quercitrin, and quercetin), biflavonoids (biapigenin and amentoflavone), naphthodianthrones [hypericin (HP) and pseudohypericin (PHP)], and phloroglucinols [hyperforin (HF) and adhyperforin (AHF)] (*3*−*6*). Several years ago, HP was thought by scientists to be responsible for the antidepressant activity of hypericum extracts (*7*). Dietary supplements of SJW in the U.S. and German markets are usually standardized to certain levels of the HP content (*8*). However, recent studies have shown that phloroglucinols, including HF and AHF (**Figure 1**), are more active antidepressants than HP (*9*−*12*). It was reported that the SJW extract containing higher amounts of HF (5%) was significantly more effective than placebo in alleviating symptoms of depression. No significant superiority over placebo could be demonstrated with the lower amounts of HF extract (*9*). It was suggested that the antidepressant property of HF is due to enhanced concentrations of monoamines and glutamate in the synaptic cleft, probably as a consequence of uptake inhibition (*13*).

More studies on the toxicology and metabolism of HF have been reported recently. Evidence has demonstrated that SJW

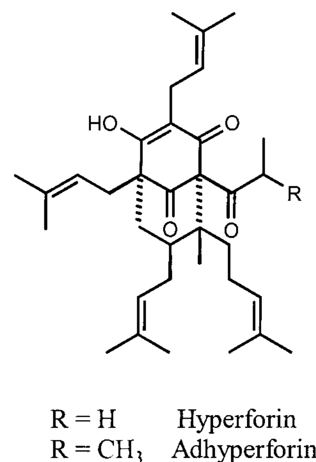

Figure 1. Chemical structures of hyperforin and adhyperforin.

dietary supplements are associated with the increase of metabolism of several co-administered drugs (*14*), such as the HIV protease inhibitor indinavir (*15*), the immunosuppressant cyclosporin (*16*, *17*), and the synthetic estrogen ethinylestradiol (*18*). These effects were explained in part by the action of HF on the pregnane X receptor system (*19*). However, another study has shown that HF is a competitive inhibitor of CYP 3A4 and a noncompetitive inhibitor of CYP 2D6 (*20*).

Most of the published analytical methods suggested using organic solvents for the extraction of bioactive components from SJW (*10*, *21*). Chatterjee et al. (*9*) reported that HF and AHF could be specifically extracted by CO_2. Improved, large-scale extraction methods with stabilizers or processing procedures were reported for production of SJW extracts with high HF

* To whom correspondence should be addressed. Tel.: 1-870-543-7400. Fax: 1-870-543-7686. E-mail: Cang@nctr.fda.gov.

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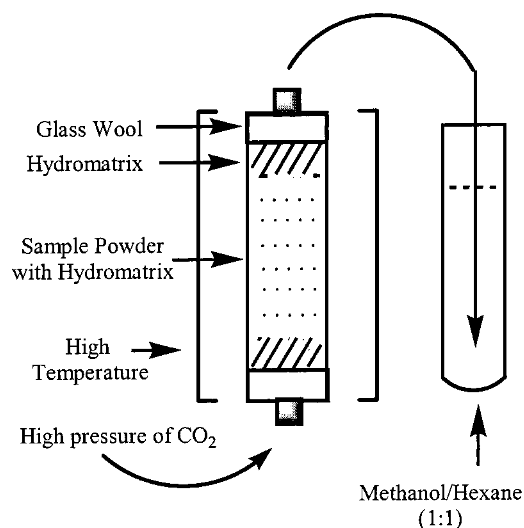

Figure 2. Simplified diagram of supercritical fluid extraction.

Table 1. Hyperforin and Adhyperforin Content of SJW Leaf/Flower Mixture Extracted by SFE Followed by HPLC Determinations

	intra-day ($n = 3$)		inter-day ($n = 4$)	
compound	mean ± SD mg/g	RSD %	mean ± SD mg/g	RSD %
hyperforin	5.02 ± 0.64	12.7	5.05 ± 0.67	13.3
adhyperforin	0.87 ± 0.09	10.1	0.90 ± 0.11	12.3

content (*22*). However, no information was provided on the supercritical fluid extraction (SFE) conditions.

Several HPLC methods have been developed to analyze various chemical constituents from these crude extracts. Chromatographic separation programs often required more than 1 h by using long gradient systems (*1*, *3*−*5*, *23*). Recently, Gray et al. (*24*) used a mixed solid-phase technique combined with an isocratic HPLC method to determine 4 compounds, i.e., HP, HF, AHF, and PHP, from flower and leaf mixtures of SJW. They used the HF calibration curve for both HF and AHF and the HP calibration curve for HP and PHP. The method required the use of two detectors, and no internal standard was used in the determination.

The objective of the present study was to develop a small, laboratory-scale SFE method for the selective extraction of HF and AHF from SJW plant materials. An isocratic HPLC/photodiode array (PDA) detection with an internal standard was also developed for the determination of HF and AHF in the SFE crude extract.

MATERIALS AND METHODS

Samples and Chemicals. Dried *H. perforatum* leaf/flower mixtures were purchased from a local natural food store in Little Rock, AR. The material was ground using a coffee grinder, passed through a 20-mesh sieve, sealed into a plastic tube, and stored at −60 °C. For protection from light degradation, the complete operation was performed under yellow light, and the plastic tube was wrapped in aluminum foil.

Benzo[*k*]fluoranthene and formic acid (97.8%) were purchased from Sigma Chemical Co. (St. Louis, MO). The liquid carbon dioxide (SFE grade) was received from Airco (Riverton, NJ). Reference standards HF and AHF were isolated from SJW leaf/flower mixture and identified by MS and NMR, and their purity was determined by HPLC/MS and HPLC/PDA (>99%) (*23*). Both HF and AHF were stored at −70 °C, and their concentrations were tested by HPLC/PDA before use. All other reagents used were purchased from J. T. Baker (Phillipsburg, NJ) and were of HPLC grade. Water was distilled, deionized, and passed through a Purification Pak (Milli-Q water purification system, Waters, Milford, MA).

HPLC Method. The HPLC instrumentation consisted of a Waters 600 pump with a 717 autoinjector and a 996 PDA detector (Waters). The instrument control and data processing were accomplished with Millenium M32 Chromatogram Manager software. The detection wavelength was set at 270 nm. The mobile phase was 92% methanol/acetonitrile (3:2) and 8% water (containing 0.1% formic acid). The flow rate was 1.0 mL/min. A LUNA C_{18} column 150 × 4.6 mm (Phenomenex, Torrance, CA) with 3-μm particle size was used.

The HPLC detector response ratios (peak area ratios) of HF and AHF to the internal standard, benzo[*k*]fluoranthene, were calculated on the basis of the sample concentrations. These corrected peak area ratios were used in the statistical analysis.

Supercritical Fluid Extraction. The SFE system (Isco, Lincoln, NE) consisted of a model 260D syringe pump and a SFX-2-10 supercritical-fluid extractor. Triplicate 1.0 g powder of leaf/flower was mixed with an equal amount of Hydromatrix (Varian, Harbor City, CA) and individually placed in a 10-mL SFE extraction cell between two layers (approximately 0.25 g each) of Hydromatrix. Both the top and bottom parts of the cell were filled with a layer of glass wool

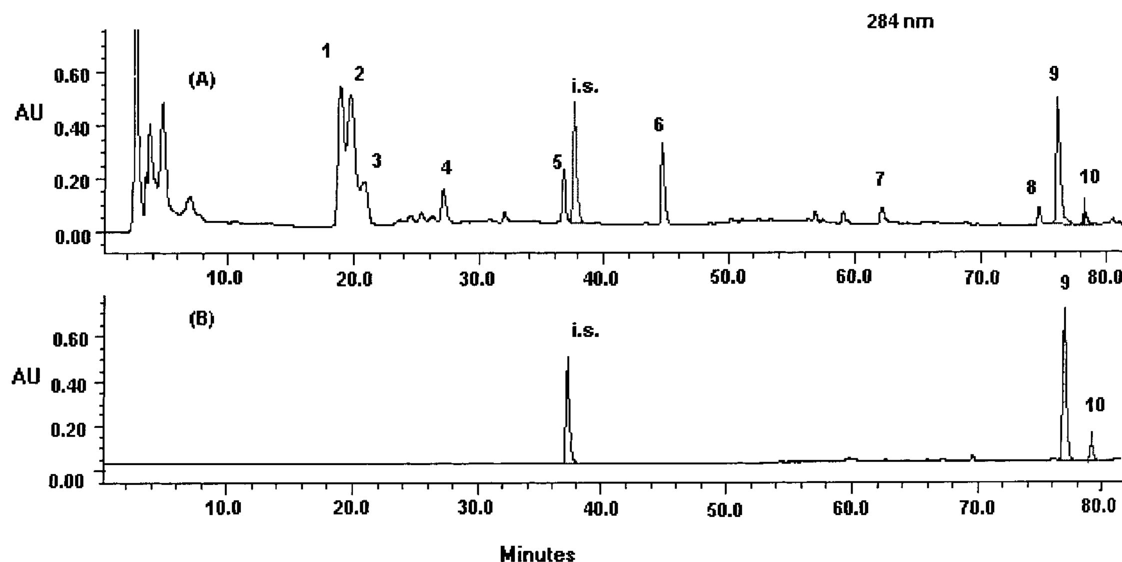

Figure 3. Gradient HPLC chromatograms of SJW extracts from (A) ultrasonic extraction and from (B) SFE extraction at 284 nm: 1 = rutin; 2 = hyperoside; 3 = isoquericitrin; 4 = quercitrin; 5 = quercetin; 6 = biapigenin, 7 = pseudohypericin; 8 = hypericin; 9 = hyperforin; 10 = adhyperforin; and i.s. = internal standard, luteolin (*25*).

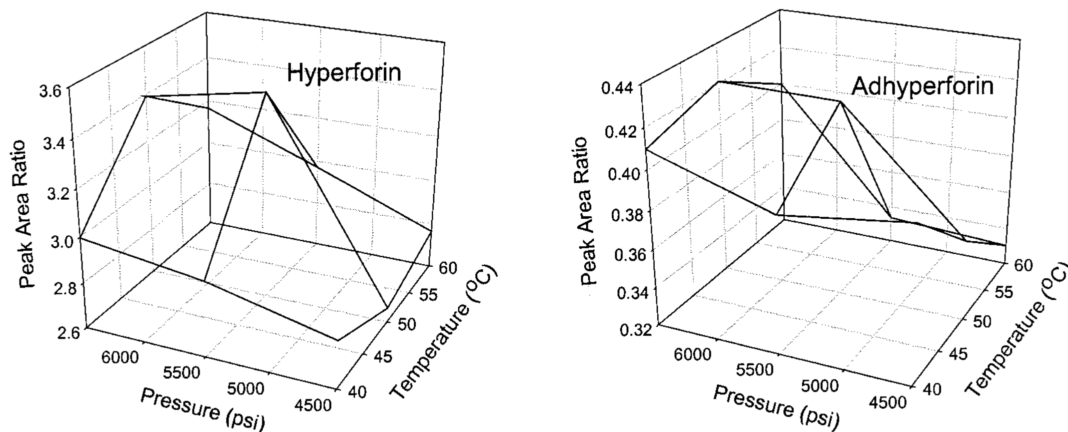

Figure 4. Effect of SFE pressure and temperature on the extraction efficiency.

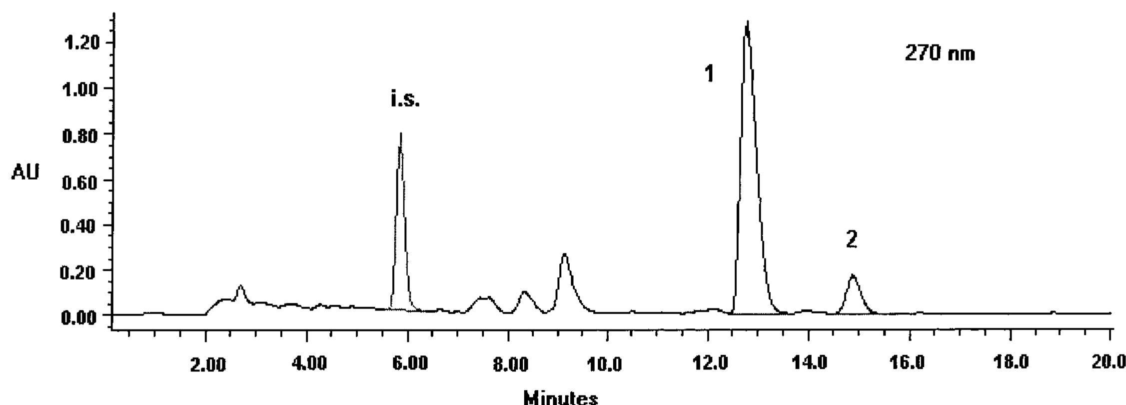

Figure 5. Typical isocratic HPLC chromatogram of St. John's wort SFE extract detected at 270 nm: i.s. = internal standard, benzo[*k*]fluoranthene; 1 = hyperforin; and 2 = adhyperforin.

Table 2. Linear Calibration Curves Derived for Hyperforin and Adhyperforin

compound	$Y = aX + b$ the linear model[a] slope (a)	intercept (b)	regression coefficient (r^2)	concentration range (mg/mL)
hyperforin	2.093	0.099	0.9986	1.00–8.00
adhyperforin	1.722	0.031	0.9948	0.050–0.400

[a] *Y*, concentration (mg/mL); *X*, peak area ratio.

(**Figure 2**). Samples were held in static extraction at 50 °C and at 3.80 × 10^4 kpa (5500 psi) for 10 min, followed by a dynamic extraction at about 1.0 min/mL for 1.5 h. The outlet temperature was set at 60 °C to prevent plugging. The extraction effluent was collected in 15 mL of methanol mixed with hexane (1:1). Another 5 mL of the solvent was added to the collection solution every 15 min during the extraction. For optimization of extraction conditions for the leaf/flower mixture, different temperatures (40, 50, and 60 °C) and pressures (3.11, 3.80, and 4.49 × 10^4 kpa, i.e., 4500, 5500, and 6500 psi, respectively) were investigated.

After the SFE procedure, the collection solution was partitioned once with 10 mL of hexane saturated with methanol. Then the methanol portion was dried under a stream of nitrogen in the dark. The residue was dissolved in 4.0 mL of methanol containing 0.10 mg/mL benzo-[*k*]fluoranthene as an internal standard. A 20-μL portion was used for HPLC analysis.

RESULTS AND DISCUSSION

Optimum SFE Conditions. HF and AHF are highly lipophilic and rather unstable when exposed to heat and light either in dry form or in solution (*23*). Orth and associates (*21*) reported that the total loss of HF during the isolation procedure by turbo liquid−liquid extraction and HPLC semipreparation was 85%. The high cost of HF and the unavailability of AHF from commercial sources were limiting factors in further investigations on pharmacology and toxicology of these two compounds.

HF and AHF are highly soluble in hexane and thus they were selectively extracted by the SFE method, but other constituents in St. John's wort such as flavonoids and naphthodianthrones were not, similar to the report of Chatterjee et al. (*9*). **Figure 3** shows a comparison of the HPLC chromatograms of ultrasonic extraction (*25*) and SFE recorded at 284 nm, the determination wavelength used by Cui et al. (*25*). The use of a supercritical solvent, CO_2, has several advantages related to its solvent power and the ease of solvent removal. CO_2 has a low latent heat of evaporation and a high volatility, which allow one to obtain extracts with very low residual solvent levels without the use of high temperature and, therefore, without the possibility of degradation of unstable components. As a solvent, supercritical CO_2 emulates hexane in densities at low pressures and methylene chloride−acetone−chloroform at higher pressures. The phase will not be condensed regardless of the pressure increase (*26*). Within this mode, the physical properties of the super-

critical CO_2 − liquidlike density, intermediate diffusivity, gaslike viscosity, and gaslike surface tension − provide a selective extraction.

It is important to maximize the contact of the supercritical fluid solvent with the sample material in order to enhance the efficiency of SFE extraction. Several variables that influence the solvent contact with sample material include flow rate, SFE time, and SFE mode (static with no follow-through or dynamic with follow-through) (*26*). Holcomb and associates (*27*) reported that a 10- to 20-min static extraction prior to dynamic extraction improved the extract recoveries in SFE extraction of aflatoxins. In the present study, static extraction longer than 10 min did not increase extraction efficiency. At the flow rate 1 mL/min (60 °C and at 3.80×10^4 kpa), about 83.2% of the total extractable HF and 88.3% extractable AHF were extracted in the first hour, and about 95.3% extractable HF and 97.2% extractable AHF were extracted in the first 1.5 hour. No more than 4% HF and AHF were extracted within another hour after the 1.5-h dynamic extraction. Thus, 10-min static extraction followed by 1.5-h dynamic extraction was used for all samples.

In SFE, pressure and temperature are the two most important instrumental parameters. Together they define the density of the supercritical CO_2 and affect solubility of analytes, which in turn affects SFE yields. Moreover, temperature and pressure have different effects on the density of the supercritical fluid, which increases as the pressure increases and decreases as the temperature increases. Variations in the combination of pressure, temperatures, and modifiers distinctly affect supercritical fluid solvent powers (*26*). In our study, temperature and pressure were evaluated for optimization of the extraction procedure. The results are shown in **Figure 4**. It was concluded that the optimal SFE system was at 50 °C and at 3.80×10^4 kpa (5500 psi). Higher temperature or higher pressure did not increase the extraction efficiency.

Both hexane and methanol are miscible with liquid CO_2 (*28*), and the extraction efficiencies of *n*-hexane and methanol for hyperforin were 94% and 92.5%, respectively, compared with that of petroleum ether (*21*). It was reported that HF and AHF were more stable in methanol (*29*), and the evaporation rate of methanol was much slower than hexane. Thus, methanol mixed with hexane (1:1) was selected as the collection solvent.

In some SFE operations, a modifier (such as methanol or acetonitrile) was used to aid the penetration of supercritical fluid in certain matrixes and to promote rapid and efficient yields. In this study, methanol (100 μL) was tested as a modifier and added to the bottom layer of Hydromatrix in the SFE cell before SFE extraction. With the modifier, only 5.79 ± 0.42 mg HF and 0.90 ± 0.09 mg AHF were extracted from 1.0 g of leaf/flower mixture. Compared to the data in **Table 1**, no significant change in the extraction of HF and AHF was obtained with the modifier, but more polar impurities appeared. Thus, no modifier was used in further investigations.

The SFE extract in the collection solvent was further partitioned with hexane in order to remove interference without affecting HF and AHF recoveries. The variations of intra-day and inter-day extraction and analysis are shown in **Table 1**.

Compared with the data obtained by ultrasonic extraction of SJW leaf/flower (*25*), SFE extracted about 60.8% HF and 60.4% AHF from samples containing 8.26 mg/g HF and 1.44 mg/g AHF as obtained by the ultrasonic method. The SFE extraction efficiencies for HF and AHF from the dietary supplements, such as capsules or tablets, were much lower than those of the ultrasonic extraction method. Thus, the present SFE method is useful for the small scale, qualitative isolation of HF and AHF from SJW leaf/flower; however, it does not appear to be suitable for quantitative determination of HF and AHF, especially from capsules or tablets.

HPLC Methods. As SFE can selectively extract phloroglucinols (HF and AHF) from SJW without co-extracting most other compounds (flavonoids and naphthodianthrones), a simple and sensitive isocratic HPLC method was developed to analyze the SFE extract (**Figure 5**). The chemical structures of HF and AHF are unique, unstable, and very expensive (AHF is not available from commercial sources). It is cost-effective to use an internal standard, such as benzo[*k*]fluoranthene, for the analysis of SFE extracts, although its structure is not similar to HF and AHF. Under the chromatographic conditions used in this study, HF, AHF, and internal standard were well separated with baseline resolution and their retention times were 12.8, 14.9, and 5.9 min, respectively. The total run time was less than 20 min. The identification of HF and AHF peaks was made by matching the retention times with reference standards and by HPLC/PDA spectra. Standard curves derived for HF and AHF with the regression equations and correlation coefficients were constructed and are shown in **Table 2**. Good linear relationships were obtained in the range assayed.

CONCLUSIONS

An optimized, small-scale SFE method was developed for the selective extraction of HF and AHF from St. John's wort leaf/flower mixture. However, its extraction efficiency was inadequate for other types of St. John's wort dietary supplements, such as capsules or tablets. An isocratic HPLC method was developed for the determination of HF and AHF in SFE extract. The SFE and isocratic HPLC method provide a rapid and convenient means to isolate and monitor the concentrations of HF and AHF, the two expensive and important active components in SJW. The methods developed are potentially useful in further SJW research.

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