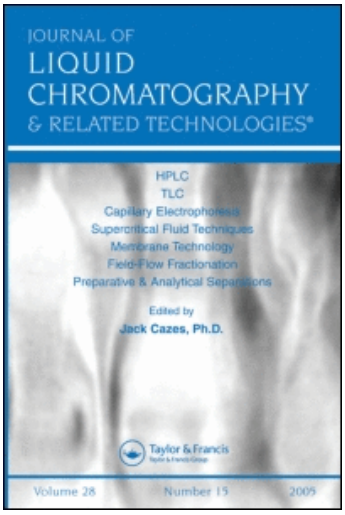

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Pyo, Dongjin , Yoo, Jisun and Surh, Jeonghee(2009) 'Comparison of Supercritical Fluid Extraction and Solvent Extraction of Isoflavones from Soybeans', Journal of Liquid Chromatography & Related Technologies, 32: 7, 923 — 932
To link to this Article: DOI: 10.1080/10826070902787351
URL: http://dx.doi.org/10.1080/10826070902787351

Journal of Liquid Chromatography & Related Technologies®, 37: 923–932, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070902787351

Comparison of Supercritical Fluid Extraction and Solvent Extraction of Isoflavones from Soybeans

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Abstract: Supercritical fluid extraction (SFE) with supercritical carbon dioxide and solvent extraction of isoflavones from soybeans were investigated. Extraction efficiencies under several different extraction conditions were examined. For SFE, pressure, temperature, organic modifier content, were optimized. The best extraction conditions were 55°C, 100 bar, and 7.5% ethanol modifier. Extraction recoveries of daidzein (97.3%) and genistein (98.0%) were obtained. For the solvent extraction, with 30 mL of 1 N HCL and 80% ethanol, daidzein of 676.3 μg/g and genistein of 659.9 μg/g were extracted. Data shows that SFE gives a little lower isoflavones recovery compared to conventional extraction with organic solvents. However, the SFE method has several advantages over the solvent extraction method. Sample handling steps are minimized, thus reducing possible losses of analytes and saving analysis time. No cleanup steps are employed, and no organic solvent extractions are involved in this method.

Keywords: Isoflavone, Solvent extraction, Supercritical fluid extraction

INTRODUCTION

In recent years, there has been an increased interest with regard to dietary phytoestrogens, especially isoflavones, among the public and in the

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medical community because of their potential role in promoting health.[1–3] The most abundant food sources of isoflavones are soybean and soybean products.[4] Soybean isoflavone has been examined for its beneficial effects on clinical and metabolic variables in animals and humans. Soybean isoflavone has been reported to act as a selective estrogen receptor modulator,[5] possess potential for antioxidant activity,[6–8] and lower blood cholesterol levels.[9–11] The chemical structures of soybean isoflavones, daidzein and genistein are shown in Figure 1.

Phytoestrogens are natural substances found in plants and are well known to regulate the endocrine system. Phytoestrogens are generally divided into isoflavones, coumestans, and lignins.[12] Among them, isoflavones and coumestans are known as the most widely distributed phytoestrogens in higher plants.[13]

Extraction with supercritical fluids as solvents have received wide attention recently. A number of potential advantages including more rapid extraction rates, more efficient extractions, increased selectivity, potential for combined analyte fractionation in conjunction with extraction, are possible with supercritical fluid extraction (SFE).

These advantages of SFE accrue from the properties of a solvent at temperatures and pressures above its critical point. At elevated pressure this single phase will have properties, which are intermediate between those of the gas and the liquid phases and are dependent on the fluid composition, pressure, and temperature. The compressibility of supercritical fluids is large, just above the critical temperature, and small changes in pressure result in large changes in density of the fluid.[14] The density of a supercritical fluid is typically 10^2–10^3 times that of the gas. Consequently, molecular interactions increase due to shorter intermolecular distances. However, the diffusion coefficients and viscosity of the fluid, although density dependent, remain more like that of a gas.[14] The 'liquid like' behaviour of a supercritical fluid results in greatly enhanced solubilizing capabilities compared to the corresponding liquid. These properties allow similar solvent strengths to liquids, but with greatly improved mass transfer properties, which provide the potential for more rapid extraction rates and more efficient extraction due to better penetration of the matrix.

Supercritical CO_2 extraction has been commercially applied to caffeine removal from green coffee beans[15] and extraction of PAHs in a roadside soil.[16] Other applications have been described in many articles.[17,18]

This study describes an investigation conducted to evaluate the applicability and efficiency of SFE methods for the extraction of isoflavones from soybeans. As a control, the conventional solvent extraction of isoflavones from soybeans was also conducted. In our laboratory, in the past, aqueous acetic acid modified CO_2 was used for the extraction of microcystins from cyanobacteria[19] and aqueous methanol was also used as a modifier.[20] In this study, we tried a new organic solvent,

ethanol as a cosolvent, so that we could obtain a fairly high extraction efficiency of isoflavones from soybeans.

EXPERIMENTAL

Reagents and Chemicals

Soybean samples were collected from local farms within the region of Gangwon-do (Korea) in the spring of 2008. The standard of isoflavones (daidzein and genistein) were purchased from Sigma (St. Louis, MO, USA). Supercritical fluid extractions were performed with carbon dioxide, SFC grade (Scott Specialty Gases, Plumsteadville, PA). All solvents were HPLC grade from Aldrich (Milwaukee, USA).

Organic Solvent Extraction

A soybean powder sample (1 g) was extracted with 30 mL of 1 N HCL refluxing for 1.5 hr. The refluxing temperature was 98–100°C. After the first refluxing, the following refluxing was performed with 30 mL of 80% ethanol which contained 0.05% BHT for 30 min, then filtered through Whatman No. 42 filter paper and PVDF syringe filter. The residue was dissolved in 5 mL of methanol and applied for the HPLC analysis.

Supercritical Fluid Extraction

Supercritical fluid extractions of isoflavones from soybeans were performed using a JASCO (Tokyo, Japan) LC-900 SFE system. The schematic diagram of the system is shown in Figure 2. This system consisted of three sections; fluid delivery, extraction, and collection. The fluid delivery section included two pumps, which delivered liquid carbon dioxide and a modifier solvent separately. In the extraction section, supercritical fluid extraction was performed with carbon dioxide modified ethanol. The collection section included a back-pressure regulator, which kept the pressure of an extraction vessel at a desired value.[21] The effluent flowing through the back-pressure regulator reduced its pressure to atmospheric pressure and thereby, solutes in the effluent reduced their solubility to virtually zero. In this way, the solutes were deposited and collected in a collection vessel. Since we used ethanol modified CO_2 as a extracting solvent, the extracts were collected in a liquid solvent in the collection vessel. The detailed list of components of the system are given in the Figure 2 caption.

High Performance Liquid Chromatography (HPLC)

HPLC analysis was performed using a Beckmann 116 pump (SYSTEM GOLD Programmable Solvent Module 126). A Waters Spherisorb ODS2 (150×4.6 mm, 5 μm) column was used. HPLC conditions were setup using a constant flow at 1 mL/min and UV detector was set at 254 nm. The mobile phase consisted of solvent A, acidified water (0.1% acetic acid), and solvent B, acidified acetonitrile (0.1% acetic acid). The initial mobile phase condition was 85% solvent A. A gradient was set to increase solvent B from 15 to 35% within 50 min. After 1 min at a constant ratio, the pumps were reset to starting conditions in four minutes. All samples were filtered through a 0.5 μm fluoropore membrane filter (Millipore, Bedford, MA, USA) prior to injection (20 μL) into the column. Isoflavone identification was performed by comparing retention times and UV spectra of purified samples to the pure standard. A further confirmation was performed co-injecting samples together with the isoflavone standard. Isoflavone quantification was carried out by comparing peak areas of investigated samples to the calibration curve of authentic standards. Retention times recorded for isoflavone in these conditions were 26.5 min of daidzein and 30.2 min of genistein.

RESULTS AND DISCUSSION

Prior to supercritical fluid extraction, solvent extractions of isoflavones from soybeans were performed. Of the soybean powder sample, 1 g was extracted with 30 mL of 1 N HCL with refluxing for 1.5 hr. The refluxing temperature was 98–100°C. After the first refluxing, the following refluxing was performed with 30 mL of 80% ethanol or standing or stirring with 80% ethanol. Thirteen solvent extraction experiments with different experimental conditions were made with the soybean samples. These experimental conditions and extraction results are resumed in Table 1. Experimental results resumed in Table 1 show that the highest recoveries of daizein and genistein were obtained with 30 mL of 1 N HCL, 1.5 hr reflux and 80% EtOH, 30 min reflux. Daizein and genistein were extracted, 676.3 μg and 659.9 μg from 1 g of soybean powder.

For the supercritical fluid extraction of isoflavones from soybeans, the suitable extraction fluid should be used. The main limitation of the most often used supercritical fluid, CO_2, is its limited ability to dissolve polar molecules, even at very high densities. Other possible neat fluids for such purposes often are reactive, flammable, or toxic. Alternatively, the characteristics of the supercritical fluid mobile phase can be varied by the addition of miscible compounds to the supercritical CO_2. The isoflavones included in this study is sparsely soluble in neat CO_2. For

Table 1. Efficacy of solvent extractions of isoflavones by various experimental conditions

Experimental condition	Isoflavone concentration (μg/g)	
	Daidzein	Genistein
1 N HCl (30 mL), 2 hr Reflux, 80% EtOH, 1 hr standing	650.5	545.8
1 N HCl (20 mL), 2 hr Reflux, 80% EtOH, 1 hr standing	615.3	506.7
1.25 N HCl (30 mL), 2 hr Reflux, 80% EtOH, 1 hr standing	671.8	520.2
1 N HCl (30 mL), 1.5 hr Reflux, 80% EtOH, 1 hr standing	418.9	202.6
1 N HCl (30 mL), 2 hr Reflux, 80% EtOH, 1 hr Reflux again	681.6	264.2
1 N HCl (30 mL), 1 hr Reflux, 80% EtOH, 1 hr Reflux again	588.1	544.6
1 N HCl (30 mL), 2 hr Reflux, 80% EtOH, 30 min Refluxing	691.1	566.5
1 N HCl (30 mL), 1.5 hr Reflux, 80% EtOH, 30 min Refluxing	676.3	659.9
1 N HCl (30 mL), 1.5 hr Reflux, 80% EtOH, 20 min Refluxing	689.7	558.5
1 N HCl (30 mL), 1.5 hr Reflux, 80% EtOH, 1 hr stirring	658.8	485.6
1 N HCl (30 mL), 1.5 hr Reflux, 80% EtOH, 2 hr stirring	655.9	557.5
1 N HCl (30 mL), 1.5 hr Reflux, 80% EtOH, 3 hr stirring	672.5	553.6
1 N HCl (30 mL), 1.5 hr Reflux, 80% EtOH, 6 hr stirring	224.0	266.6

example, when neat CO_2 is used as the extraction fluid at 55°C and 100 bar, no isoflavones could be extracted from dried soybean powder. However, with ethanol modified CO_2, the extraction of isoflavones was successful. In supercritical fluid extraction, the extraction of an analyte depends on its distribution between the supercritical fluid and the sorptive sites in the sample matrix. In general, for predicting optimal extraction conditions, one must have two considerations in mind: the ability of the supercritical fluid to compete with the analytes for the sorptive sites and the solubility of the analytes in the supercritical fluid. The latter usually appears to be more important. From Figure 1, isoflavones contain several polar groups (−OH). The poor extraction result with neat CO_2 is probably caused by the fact that isoflavones consist of fairly polar

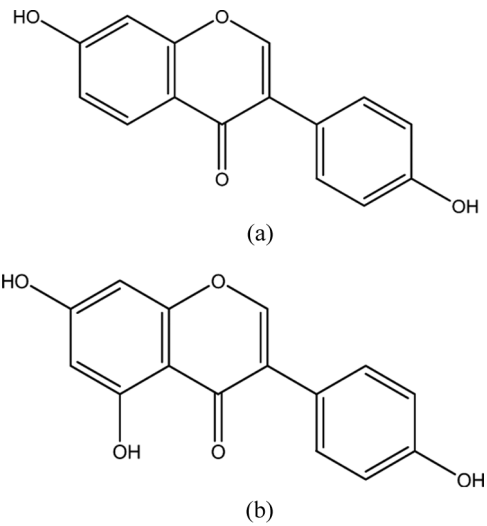

Figure 1. Chemical structure of (a) Daidzein (b) Genistein.

functional groups. The use of co-solvents can have a profound effect on increasing the solubility levels of polar solutes in supercritical fluids.

In this study, our experimental results have indicated that 7.5% ethanol was the most suitable co-solvent for the supercritical fluid extraction

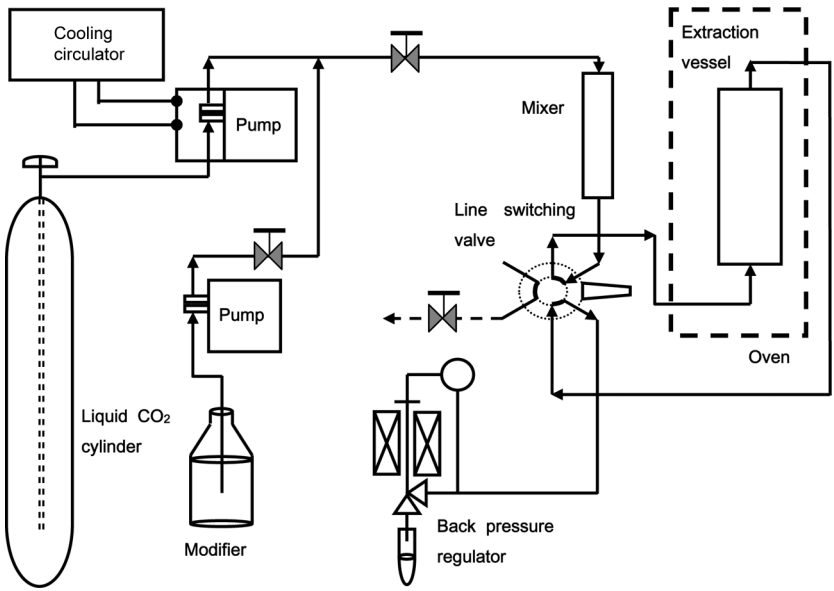

Figure 2. Schemetic flow diagram of SFE modular system.

Table 2. Supercritical fluid extraction of isoflavones using various temperatures. Experimetal condition: 120 min extraction at 100 bar, CO_2 flow 2.0 mL/min and modifier flow 0.2 mL/min. RSDs based on triplicate extractions under each condition

Temperature, °C	Extraction of genistein (%)	Extraction of daidzein (%)
45	90.5 ± 4	88.5 ± 3
55	97.3 ± 3	98.0 ± 3
60	87.8 ± 4	82.7 ± 3

of isoflavones from soybeans. The co-solvent flow rate was 0.2 mL/min and the supercritical CO_2 fluid flow rate was 2.0 mL/min. Therefore, the most suitable medium for the supercritical fluid extraction of isoflavones was a mixed fluid (92.5% CO_2 and 7.5% ethanol). When 7.5% ethanol was used as a co-solvent at 55°C and 100 bar, 97.3% of daidzein and 98.0% of genistein were extracted. It is important to find the optimum SFE operating conditions which would result in the most efficient extraction of isoflavones from soybeans. In particular, the pressure and temperature of the supercritical fluid are the two most important parameters to be optimized for the most SFE experiments. To find the optimum extraction temperature, the temperature of the extraction vessel was varied from 45°C to 65°C (Table 2). The best extraction efficiency is shown at 55°C, therefore, the temperature used in this study was 55°C. The solvating strength of a supercritical fluid is related to its density, a parameter primarily dependent upon pressure. For this study, the extraction pressure was increased from 80 bar to 120 bar at intervals of 10 bar (Table 3). The extraction efficiency increases with increasing the pressure of extracting fluid until the pressure reaches 100 bar. At higher pressures than 100 bar, the extraction efficiency decreases since some amount of

Table 3. Supercritical fluid extraction of isoflavones using various pressures. Experimetal condition: 120 min extraction at 55°C, CO_2 flow 2.0 mL/min and modifier flow 0.2 mL/min. RSDs based on triplicate extractions under each condition

Pressure, bar	Extraction of genistein (%)	Extraction of daidzein (%)
80	82.5 ± 3	83.3 ± 3
90	89.1 ± 3	90.5 ± 3
100	97.3 ± 3	98.0 ± 3
110	79.6 ± 4	76.5 ± 4
120	52.4 ± 4	50.1 ± 4

Table 4. Supercritical fluid extraction of isoflavones using different compositions of ethanol co-solvent. Experimetal condition: 120 min extraction at 55°C and 100 bar, CO_2 flow 2.0 mL/min and modifier flow 0.2 mL/min. RSDs based on triplicate extractions under each condition

Fluid phase	Extraction of genistein (%)	Extraction of daidzein (%)
91.5% CO_2 + 8.5% ethanol	80.1 ± 4	76.2 ± 4
92.0% CO_2 + 8.0% ethanol	85.8 ± 4	87.1 ± 3
92.5% CO_2 + 7.5% ethanol	97.3 ± 3	98.0 ± 3
93.0% CO_2 + 7.0% ethanol	70.2 ± 4	65.4 ± 4
93.5% CO_2 + 6.5% ethanol	50.8 ± 4	41.2 ± 5

sticky oil component was also extracted. Other compositions of ethanol were also tried to find the best extraction condition (Table 4).

The typical HPLC chromatogram of the extract by SFE is shown in Figure 3. From Figure 3, it is noticed that when the samples are extracted by SFE, many small compounds are also extracted. However, since these small compounds elute earlier than isoflavone peaks, the peaks of those compounds do not overlap with the peak of isoflavones in HPLC chromatogram.

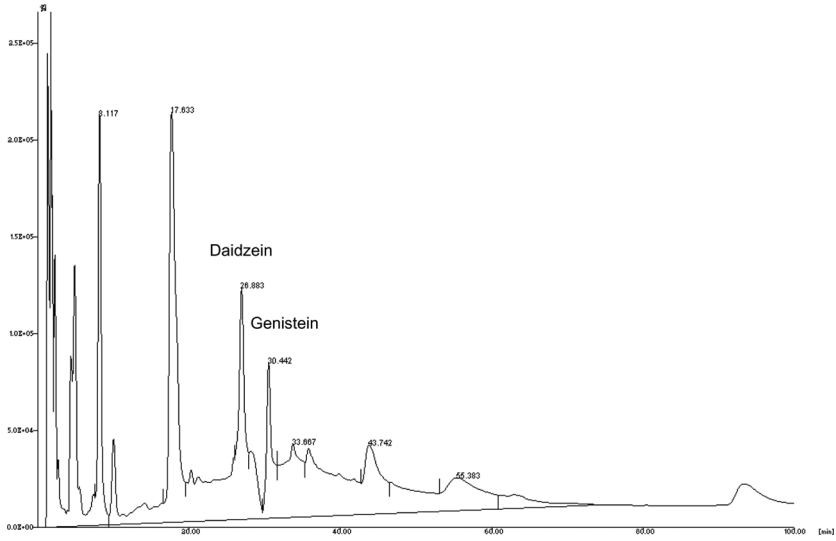

Figure 3. High performance liquid chromatogram of extracted isoflavones using SFE with a mixed fluid (92.5% CO_2, 7.5% ethanol) at 55°C and 100 bar. HPLC conditions; mobile phase [0.1% acetic acid : 99.9% water]: [0.1% acetic acid : 99.9% acetonitrile] = 85:15 to 65:35 for 50 min, flow rate 1.0 mL/min, detector 254 nm.

The SFE procedure described here has several advantages over the organic solvent extraction procedure for the analysis of isoflavones. Sample handling steps are minimized, thus reducing the possible losses of analytes and saving analysis time. No cleanup steps are employed since the SFE with ethanol modified CO_2 gives clean extracts, which can be directly analyzed with HPLC. The mixed supercritical fluid (72.5% carbon dioxide + 7.5% ethanol) system gave higher extraction efficiency than any other mixed fluid systems for the extraction of isoflavones from soybeans.

ACKNOWLEDGMENT

This study was supported by a 2007 research grant from Kangwon National University.

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Received November 22, 2008
Accepted December 12, 2008
Manuscript 6445