# EXTRACTION OF SALVIA MILTIORRHIZA USING SUBCRITICAL WATER

by

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## Introduction

Traditional Chinese medicine (TCM) originated in ancient China but has evolved over the past thousands of years and is now used all over the world. *Salvia miltiorrhiza*, also known as Danshen (Figure 1), is a root commonly used in Chinese medicine to treat various cardiovascular diseases. As it has gained more recognition, this herb is currently being investigated for its anticancer properties. In traditional Chinese medicine, the patients extract the prescribed herbs using boiling water, then consume the extracts as medication. However, the extraction temperature of approximately 100 °C used in this traditional method may not be the most effective condition to remove the active pharmaceutical ingredients (APIs) from the herb.

**Figure 1: *Salvia miltiorrhiza*[1]**

The primary components in Danshen with pharmacological importance are the tanshinones. Tanshinone IIA (Figure 2) is currently used in China and other neighboring countries to treat patients suffering from myocardial infarction (MI), angina pectoris, stroke, diabetes, sepsis, and other conditions.[2] Benefits from treatment with the herb are due to its improved microcirculatory, vasodilatory, anti-coagulant, anti-thrombotic, anti-inflammatory,

free radical scavenging, and mitochondria-protective effects.[3] It is also being researched for its anti-cancer properties; it is known to disrupt cancer cell lines and promote apoptosis of the cancerous cells.[4] Like Tanshinone IIA, Tanshinone I (Figure 2) also has anti-cancer properties. Instead of promoting apoptosis of the cancerous cells, Tanshinone I reduces the transcriptional activity of interleukin-8, the angiogenic factor involved in cancer metastasis.[5] In other words, Tanshinone I prevents the cancerous cells from growing and spreading to different areas of the body. Other anti-cancer compounds found in *Salvia miltiorrhiza* are protechualdehyde, caffeic acid, and isoferulic acid (Figure 3). Our previous study shows that higher temperatures can more efficiently extract these anticancer agents from *Salvia miltiorrhiza*.

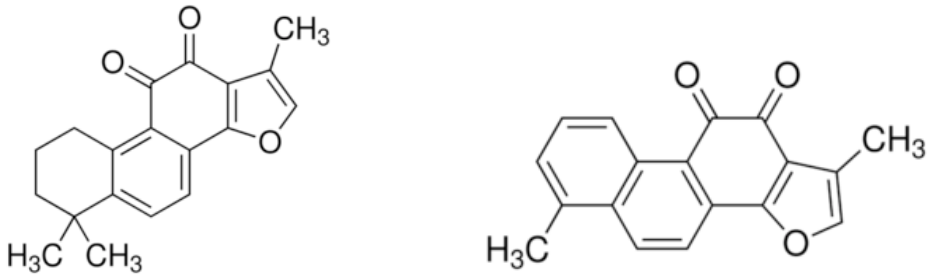


**Figure 2: Active nonpolar pharmaceutical ingredients found in *Salvia miltiorrhiza*: Tanshinone IIA[6] and Tanshinone I[7].**

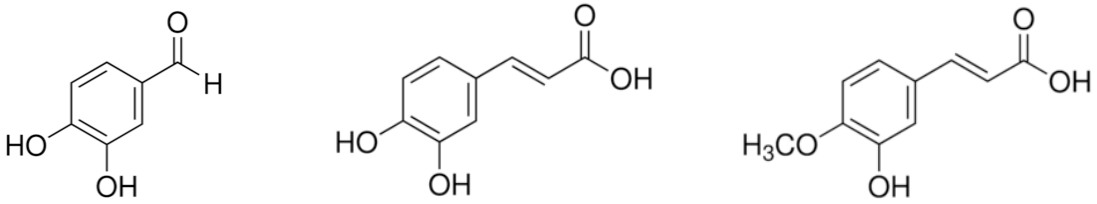


**Figure 3: Active polar pharmaceutical ingredients found in *Salvia miltiorrhiza*: protocatechualdehyde[8], caffeic acid[9], and isoferulic acid[10].**

Subcritical water extraction (SBWE) is an extraction technique that uses heated liquid water as the extraction fluid. Subcritical water is water that is heated between 100 °C and 374 °C and is applied with moderate pressures. Subcritical water is very useful in the separation field. At higher temperatures, water becomes less polar and is capable of extracting nonpolar and hydrophobic molecules due to its lowered dielectric constant.[11] At elevated temperatures, water can be used as a substitute solvent for methanol. Subcritical water extraction is a green separation technique because it is more environmentally friendly than using methanol or other organic solvents, which are used in other methods of extracting the herb. Subcritical water extraction is the best method for the extraction of this herb due to the elimination of organic solvents. The goal of this study is to determine the most efficient subcritical water extraction temperature that extracts the active pharmaceutical ingredients: protechualdehyde, caffeic acid, isoferulic acid, Tanshinone I, and Tanshinone IIA from *Salvia miltiorrhiza*.

**Experimental**

The *Salvia mitiorrhiza* root (Danshen) and deionized water were used in this work. Other chemicals used are methanol, dichloromethane, phosphoric acid, Tanshinone I, Tanshinone IIA, protocatechualdehyde, caffeic acid, and isoferulic acid. The SBWE system was homemade with an ISCO Model 100DX syringe pump and ISCO Series D pump controller. The oven for the system was taken from an Agilent 6890 gas chromatography (Model: G1530A) system. A diagram of the system used is shown in Figure 4.

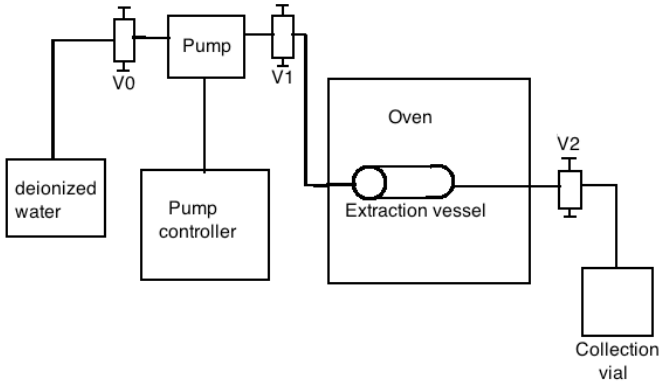


**Figure 1: Block diagram of SBWE system used for extractions.**

The extraction vessel was loaded with approximately 1.5 g of ground Danshen then filled with sand. A filter made from Kleenex was inserted in the out flow end. The extraction vessel was then connected inside the oven. Before starting the heating process, V2 was closed and the extraction vessel was pressurized by increasing the pressure in increments of 2 atm until the flow rate was no longer negative. The pressures used were 10, 12, 16, and 20 atm for 75, 100, 125, and 150 °C, respectively. Once the vessel was pressurized, V1 was closed and the heating began. The loaded extraction vessel was heated for 60 minutes with an additional equilibration time of 8, 12, 14, and 16 minutes for 75, 100, 125, and 150 °C, respectively. After heating, V2 and V1 were opened and the sample was collected at a constant flow rate of 1.0 mL/min. The sample collected in the collection vial was the water extract. The residue sample that remained in the vessel was removed and sonicated for approximately 4-5 hours with approximately 5 mL of methanol. Each extraction temperature was done in triplicate.

To simulate TCM practice of boiling the herb over the stove, approximately 1.5 g of ground Danshen was added to a 100 mL beaker with 30 mL of 18 MΩ deionized water. The

beaker was covered with a watch glass and heated to a constant boil on a hot plate for 60 minutes. The herb residue and water extract were separated and treated with the same procedure as the other samples.

Liquid-liquid extraction was performed on each water extract. Dichloromethane (2 mL) was added to each vial. Each vial was then vortexed and the nonpolar bottom layer was removed and put into a separate vial. Because dichloromethane is nonpolar, or only slightly polar, it is assumed that the tanshinones are in this layer. The remaining water layer, containing the polar compounds, was filtered to separate the remaining liquid from the solid. Methanol (1 mL) was added to each filtrate.

An internal standard solution was prepared and added to each solution. A paraben mixture, propyl 4-hydroxybenzoate (125.4 mg), was dissolved by methanol in a 50 mL volumetric flask and diluted to the mark. Then, 30 µl of this internal standard was added to each of the dichloromethane and sonication filtrate vials. 300 µl of the internal standard was added to each of the water filtrate vials.

High-Performance Liquid Chromatography (HPLC, Shimadzu Nexera UFLC system) was used to analyze each of the samples. The mobile phases used were methanol and a prepared acidified water solution. The water solution was prepared by adding 10 mL of phosphoric acid to 1 L of 18 MΩ deionized water. The samples were run at 25 °C; the condition for gradient elution is shown below in Table 1:

**Table 1: HPLC Gradient Conditions**

| Time | %MeoH | % $H_3PO_4$ in H2O | UV |
|---|---|---|---|
| 0.0 | 2 | 98 | 270 |
| 4.0 | 2 | 98 | |
| 8.0 | 10 | 90 | |
| 15.4 | | | 254 |
| 23.0 | 30 | 70 | |
| 32.0 | 60 | 40 | |
| 43.0 | 60 | 40 | |
| 49.0 | 70 | 30 | |
| 53.0 | | | 270 |
| 61.0 | 80 | 80 | |
| 62.0 | 90 | 10 | |
| 65.0 | 90 | 10 | |
| 66.0 | 2 | 98 | |
| 68.0 | 2 | 98 | |

**Results and Discussion**

Peak areas from the HPLC chromatograms of the different analytes were used in determining the concentrations of those analytes. The concentrations of the analytes found in the SBWE extract (water and dichloromethane layers) are shown in Table 2. The concentrations of the analytes found in the herb residue are shown in Table 3. The concentrations are shown in micrograms of analyte per grams of herb loaded into the column.

**Table 2: Concentration of APIs Found in SBWE Extract, µg/g (%RSD)**

| Analyte | TCM Replicate | 75 °C | 100 °C | 125 °C | 150 °C |
|---|---|---|---|---|---|
| Protocatechualdehyde | 158.5 (80.1) | --- | 231.7 (175.3) | 867.7 (29.0) | 876.2 (57.2) |
| Caffeic Acid | --- | 117.1 (340.2) | --- | 9.6 (18.2) | 11.2 (121.8) |
| Isoferulic Acid | 7.0 (57.2) | 10.4 (92.0) | 2.8 (34.1) | 138.0 (33.0) | 37.5 (46.1) |
| Tanshinone I | 0.75 (29.8) | 0.52 (32.2) | 0.81 (43.2) | 2.9 (89.5) | 3.1 (143.8) |
| Tanshinone IIA | 1.5 (71.7) | 1.3 (45.6) | 1.8 (59.3) | 2.2 (44.2) | 0.36 (78.8) |

--- indicates values that could not be quantified

**Table 3: Concentration of APIs Found in Herb Residue after SBWE, μg/g (%RSD)**

| Analyte | TCM Replicate | 75 °C | 100 °C | 125 °C | 150 °C |
|---|---|---|---|---|---|
| Protocatechualdehyde | 195.44 (69.8) | --- | 110.3 (87.7) | 261.0 (79.5) | 226.4 (19.1) |
| Caffeic Acid | --- | --- | 13.8 (111.0) | 9.6 (142.5) | 0.32 (135.9) |
| Isoferulic Acid | 6.9 (23.3) | 0.34 (155.6) | 12.7 (105.7) | 148.7 (138.9) | 7.1 (49.7) |
| Tanshinone I | 39.1 (15.7) | 93.1 (92.1) | 70.9 (79.5) | 138.5 (79.9) | 15.2 (50.6) |
| Tanshinone IIA | 239.1 (21.1) | 611.1 (76.2) | 598.2 (49.6) | 512.3 (61.4) | 35.1 (30.8) |

--- indicates values that could not be quantified

Based on the concentration of each analyte obtained at each temperature, the extraction efficiency of the analytes in the SBWE extract increased significantly at 125 °C, with the exception of caffeic acid. The concentrations of caffeic acid show degradation with increasing temperature. As shown in Table 2, the concentration of analytes found in the herb residue was greatest at 125 °C, with the exception of Tanshinone IIA. Although extraction at 150 °C shows promising quantities of some of the analytes, the degradation of the ingredients in the herb was severe and such a high temperature was not ideal to work with. The relative standard deviation values indicate significant error in the triplicate experiments, which could void the significance of the results. More extractions and further analysis will need to be done in the future to ensure legitimacy of the results.

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