# One-Step Purification of 3,4-Dihydroxyphenyllactic Acid, Salvianolic Acid B, and Protocatechualdehyde from *Salvia miltiorrhiza* Bunge by Isocratic Stepwise Hydrogen Bond Adsorption Chromatography on Cross-Linked 12% Agarose

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## Abstract

**Three major active components of the traditional Chinese medicinal herb *Salvia miltiorrhiza* Bunge, 3,4-dihydroxyphenyllactic acid, salvianolic acid B, and protocatechualdehyde, are separated and purified from a crude water extract in one step by isocratic hydrogen bond adsorption chromatography on cross-linked 12% agarose (Superose 12 HR 10/30). Separation is achieved by stepwise elution with mobile phases composed of mixtures of ethanol and acetic acid: 0–50 mL, 5% ethanol, 5% acetic acid; 50–100 mL, 20% ethanol, 20% acetic acid; and 100–200 mL, 30% ethanol, 30% acetic acid. The 3,4-dihydroxyphenyllactic acid is obtained with a purity of 97.3% and with a recovery of 88.1%. The corresponding figures for protocatechualdehyde are a purity of 99.4% with a recovery of 90.7%, and for salvianolic acid B a purity of 90.4% with a recovery of 50.3%, respectively. At a sample load of 40 mg crude extract dissolved in 0.5 mL mobile phase (corresponding to a load of 1.6 mg/mL gel), a 3,4-dihydroxyphenyllactic acid purity of approximately 94% with a recovery of 80.2% is obtained.**

## Introduction

*Salvia miltiorrhiza* Bunge, a popular traditional Chinese medicinal plant, has been used extensively for the treatment of coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrhea and neuroasthenic insomnia, and cytotoxicity against human tumor cell lines (1–3). The early studies on the chemical constituents of Salvia were mainly confined to the diterpenoids and tanshinones. In recent years, much attention has been directed to the biologically active water-soluble components (e.g., polyphenols) in dried root decoctions. These studies, particularly in China, have led to the isolation and identification of a host of caffeic acid derived metabolites, many of which possess a variety of biological activities including antioxidant, antiplatelet, antitumor, and antiviral activity (4–5). 3,4-Dihydroxyphenyllactic acid, also known as danshensu (a Chinese word literally meaning the element of Danshen), and salvianolic acid B (lithospermic acid B) are considered the major active polyphenolic components in *Salvia miltiorrhiza*. Thus, 3,4-dihydroxyphenyllactic acid was first isolated from extracts of this herb, and found to scavenge the free oxygen radicals and to be a coronary vasodilator (1). Salvianolic acid B possesses significant scavenging effects on oxygen free radicals and has shown protective effects on heart and brain injuries induced during ischemia-reperfusion (6). Protocatechualdehyde (protocatechuic aldehyde) is normally used as a reference component in the analysis of related preparations of *Salvia miltiorrhiza*.

Decoctions have been the most popular preparations of traditional Chinese medicine (TCM) for more than two thousand years. It has long been implied that water-soluble components are very important active parts of TCM. However, the current separation and purification techniques and methods available for water-soluble compounds with high polarity are very limited. The traditional method for the purification of 3,4-dihydroxyphenyllactic acid is extracting the powdered dried root of *Salvia miltiorrhiza* with water followed by precipitation with ethanol. This method is still popular for large-scale preparation of injectable 3,4-dihydroxyphenyllactic acid. Macroporous resin chromatography is another standard separation method (7). However, the purity of 3,4-dihydroxyphenyllactic acid obtained using either of these two methods is not satisfactory. Preparative high-performance liquid chromatography (HPLC) yields a high purity product. However, the production cost is very high and the sample has to be subject to cautious pretreatment, and, thus,

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HPLC is less advantageous for scaling-up in the TCM area (8). It has been reported that high-speed counter current chromatography (HSCCC) is an efficient method for the separation and purification of polyphenols of *Salvia miltiorrhiza* (9–11), including salvianolic acid B, salvianolic acid E, rosmarinic acid, and lithospermic acid, giving high purity and high yield. So far, purification of 3,4-dihydroxyphenyllactic acid by HSCCC has not been reported. The major disadvantage of centrifugal partition chromatography and HSCCC is the difficulty of scaling-up. So, new techniques and methods are needed for the preparative separation and purification of 3,4-dihydroxyphenyllactic acid and other water-soluble components in TCM. In one report, oligo-β-cyclodextrin substituted agarose gel media was used for the separation and purification of water-soluble *Salvia miltiorrhiza* components (12).

Recently, it has been shown (13) that a variety of polyphenolic substances can be separated on a column packed with heavily cross-linked 12% agarose gel particles (Superose 12 HR 10/30). This report confirms that this column can also be used for the separation and purification of several components in crude water extracts of *Salvia miltiorrhiza*.

A major advantage of cross-linked agarose gel media, besides its high selectivity for polyphenols and scaleability, is its chemical stability, allowing a range of harsh cleaning regimes, including 0.5M NaOH, to be applied for its regeneration. Thus it is possible to separate and purify polyphenols in one step from crude extracts of TCM with high yield and high purity. With regular cleaning, the media can be used reproducibly for more than 50 cycles at high loading.

## Material and Methods

### Equipment

The HPLC system (10 Avp, Shimadzu, Japan) was composed of two pumps, UV detector, oven, system controller, two sample loops, and class-vp workstation. One sample loop was 20 µL for high-pressure analysis and the other was 500 µL for low-pressure separation and purification. The analytical column used was Ultrasphere $C_{18}$ (250 × 4.6 mm i.d., 5 µm, Shimadzu, Japan). The preparative separation column used was Superose 12 HR 10/30 containing 10 µm average particle diameter cross-linked 12% agarose beads (GE Healthcare, Uppsala, Sweden).

### Reagents

Analytical-grade ethanol and acetic acid were obtained from Atoz Fine Chemicals Co. Ltd. (Tianjin, China). Methanol of HPLC grade was obtained from Merck AG (Darmstadt, Germany). Acetic acid was obtained from Concord Tech Co. Ltd. (Tianjin, China). All aqueous solutions were prepared with water produced by the Milli-Q system (Millipore, Bedford, MA). Crude water extracted powder of *Salvia miltiorrhiza* Bunge was a gift from Dalian Institute of Chemical Physics, Chinese Academy of Sciences (Dalian, China). The original *Salvia* species was supplied by Tong Li Medicinal Plant Company (Anguo, Hebei, China). The family name of the analyzed *Salvia* species is *labiate*. The plant species was identified at the Institute of Traditional Chinese Medicine, China Academy of Medical Sciences. The voucher specimen is deposited at the Dalian Institute of Chemical Physics, Chinese Academy of Sciences.

Reference standards of 3,4-dihydroxyphenyllactic acid, salvianolic acid B, and protocatechualdehyde with > 98% purity were obtained by the State Food and Drug Administration of China (Beijing, China). Their structural formulae are shown in Figure 1.

### Adsorption chromatography of a crude water extract of *Salvia miltiorrhiza* Bunge

Amounts in the range 16–100 mg crude water extract of *Salvia miltiorrhiza* were dissolved in 1.0 mL solvent mixture of 5% ethanol and 5% acetic acid. The dissolution was assisted by ultrasonication. All samples were pre-filtered using 0.22 µm pore size nylon membranes to remove possible particles before injection to the chromatographic columns. The injection volume was 0.5 mL and the flow rate used was 0.9 mL/min. The effluent was monitored at 280 nm on a Shimadzu HPLC chromatography system. Before sample application, the Superose 12 HR 10/30 column was equilibrated with two column volumes of the first mobile phase, a mixture containing 5% ethanol and 5% acetic acid. After sample application, isocratic stepwise elution was performed at the following ethanol and acetic acid concentrations: 0–50 mL, 5% ethanol, 5% acetic acid; 50–100 mL, 20% ethanol, 20% acetic acid; 100–200 mL, 30% ethanol, 30% acetic acid. After each use, the Superose 12 column was immediately cleaned in-place with 50 mL 0.05M NaOH and 50 mL 20% (v/v) ethanol

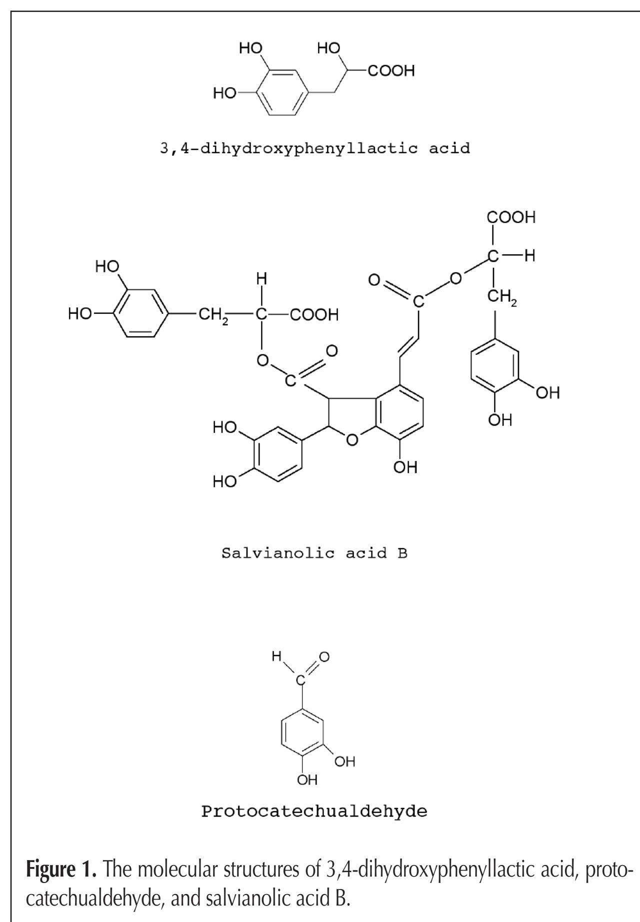


**Figure 1.** The molecular structures of 3,4-dihydroxyphenyllactic acid, protocatechualdehyde, and salvianolic acid B.

using a reversed flow through the column. After every 5th sample cycle, the column was regenerated within series: 50 mL 0.05M NaOH, 50 mL 20% (v/v) ethanol, 50 mL 50% (v/v) acetic acid, and 50 mL 20% (v/v) ethanol. The columns were stored in 20% (v/v) ethanol.

### Analysis of a crude water extract of *Salvia miltiorrhiza* Bunge and preparative chromatography fractions by HPLC

Column: Ultrasphere C-18, 250 mm × 4.6 mm, 5 µm (Shimadzu, Japan). Mobile phase: (A) methanol–water–acetic acid (10:89.2:0.8), (B) methanol–water–acetic acid (89.2:10:0.8). Gradient elution: 0–60 min, 0–60% B; flow rate: 1.0 mL/min; wavelength: 280 nm; sample volume: 20 µL.

## Results and Discussion

### Optimization of mobile phase composition

Acetic acid is known to impose a quenching effect on the adsorption of aromatic groups to tightly cross-linked polysaccharide gel media (14–16). However, there is also the possibility of hydrogen bonding to participate in the retardation of polyphenols on this highly cross-linked agarose gel (17). At an acetic acid concentration of 50%, no adsorption occurs. A tentative explanation is that the delocalized electron cloud around the acetate ion acid competes with the aromatic ring system of the polyphenols for binding to the cross-linked network structures present on the outer and inner surfaces of Superose 12 (18,19). Superose 12 contains a complex cross-linking network system created by consequtive reactions with long- and short-chain bisepoxides followed by surface substitution with epichlorohydrin (18,19).

Loading of 8 mg crude sample dissolved in water to the Superose 12 HR 10/30 column, equilibrated in water, resulted in strong adsorption. In order to control the extent of the adsorption, a variety of mobile phase compositions were investigated with the strategy that solvents with as low cost and level of pollution as possible should be chosen (e.g., mixtures of water, ethanol, and acetic acid). To this end, different concentrations of ethanol and acetic acid in distilled water were tried. None of the target substances were eluted within 100 min with 5% acetic acid or 5% ethanol (data not shown). In our experience, less favorable results were obtained when high concentration compositions, such as a mixture of 30% ethanol and 30% acetic acid, were applied from the very start of the elution. This is why a segmented isocratic elution principle with stepwise increasing solvent concentrations was applied, and resulted in reasonable resolution of three target compounds. Optimal separation conditions were achieved by applying the following stepwise isocratic elution scheme 0–50 mL, 5% ethanol, 5% acetic acid; 50–100 mL, 20% ethanol, 20% acetic acid; 100–200 mL, 30% ethanol, 30% acetic acid. The best result obtained is shown in Figure 2.

Fractions were analyzed by reversed-phase chromatography on a C-18 column. The retention times of samples of fractions A, B, and C were the same as for the three reference materials. Confirmation was obtained by mixing fraction A, B, and C with reference standards of 3,4-dihydroxyphenyllactic acid, protocatechualdehyde, and salvianolic acid B, respectively, followed by analysis on the C-18 column. Only one peak was eluted in each group (Figure 3). The delocalized electron cloud of salvianolic

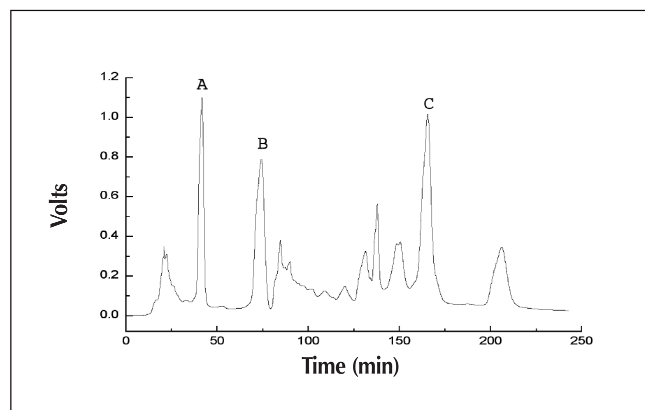


**Figure 2.** One-step adsorption chromatography with stepwise isocratic elution of a crude water extract of the root of *Salvia miltiorrhiza* Bunge on a Superose 12 HR 10/30 column. Sample: 0.5 mL of crude extract (16 mg/mL). Mobile phase: 0–50 mL: 5% ethanol, 5% acetic acid; 50–100 mL: 20% ethanol, 20% acetic acid; 100–200 mL: 30% ethanol, 30% acetic acid. Flow rate: 0.9 mL/min. Detector: UV 280 nm. 3,4-dihydroxyphenyllactic acid (A), Protocatechualdehyde (B), Salvianolic acid B (C).

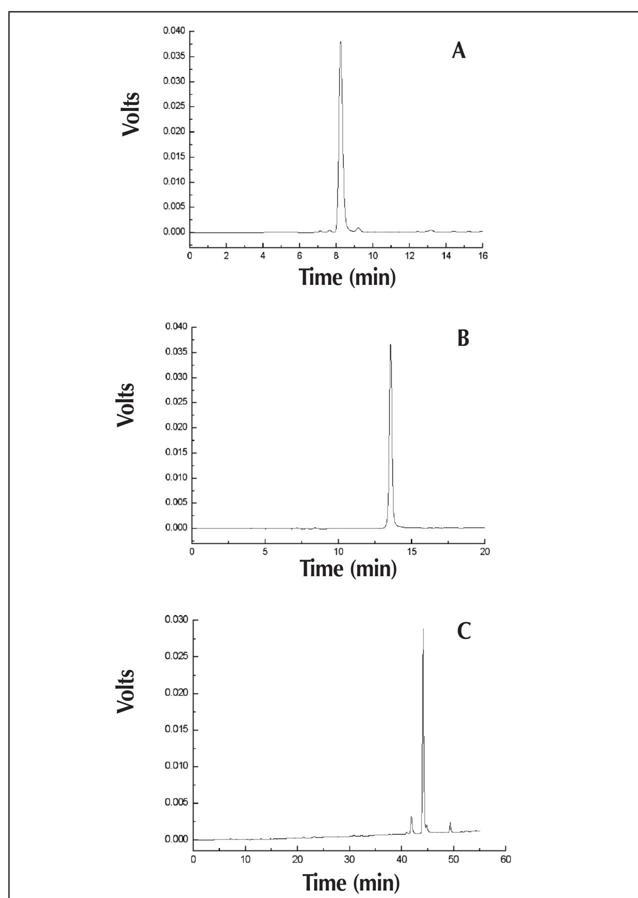


**Figure 3.** Confirmation of identity of 3,4-dihydroxyphenyllactic acid, protocatechualdehyde, and salvianolic acid B by RPC. Column: Ultrasphere C-18 reversed phase silica (250 × 4.6 mm i.d., 5 µm, Shimadzu, Japan). Mobile phase: (A) methanol–water–acetic acid (10–89.2–0.8), (B) methanol–water–acetic acid (89.2–10–0.8). Gradient elution: 0–60 min, 0-60%B. Flow rate: 1.0 mL/min. Detector: UV 280 nm. Sample volume: 20 µL.

acid B is much larger than those of the other two compounds, leading to stronger retardation. A 3,4-dihydroxyphenyllactic acid purity of 97.3% was obtained with a recovery of 88.1%. A protocatechualdehyde purity of 99.4% was obtained with a recovery of 90.7%. A salvianolic acid B purity of 90.4% was obtained with a recovery of 50.3%.

#### Effect of increased sample load

Sample loading capacity studies were performed with 8~40 mg crude water extracts of *Salvia miltiorrhiza* in 1 mL solvent mixture of 5% ethanol and 5% acetic acid on a 24 mL Superose 12 column. Focus was put on the purification of 3,4-dihydroxyphenyllactic acid, the major component in the crude extract. At a sample load of 40 mg crude extract, corresponding to 1.6 mg/mL, detection was out of limit. A 3,4-dihydroxyphenyllactic acid purity of approximately 94% was obtained with a recovery of approximately 80.2%. The application of high loads of crude extracts of natural products containing a variety of complex components inevitably leads to gel fouling and a concomitant decrease in separation efficiency. This is why, after each sample cycle, two column volumes of 0.05 M sodium hydroxide and 20% ethanol were applied to the column in reversed direction (from the bottom of the column to the top). After every 5 sample cycles, this cleaning procedure was complemented by two column volumes of 50% acetic acid. Adopting this cleaning procedure led to reproducible performance for more than 50 sample cycles of crude extract. This character of Superose 12 gives it a clear advantage over other less alkali stable separation media such as pure silica and reversed-phase silica.

## Conclusion

Adsorption chromatography on Superose 12 HR 10/30, a cross-linked 12 % agarose gel, was successfully used for the purification of the polyphenols 3,4-dihydroxyphenyllactic acid (purity of 97.3%, recovery of 88.1%), salvianolic acid B (purity of 90.4%, recovery of 50.3%), and protocatechualdehyde (purity of 99.4%, recovery of 90.7%) in one step directly from a crude water extract of the herb *Salvia miltiorrhiza*. Stepwise isocratic elution was performed with increasing concentrations of a mixture of ethanol and acetic acid. At a crude sample load of 40 mg in 0.5 mL, a 94% pure 3,4-dihydroxyphenyllactic acid could be obtained with a recovery of 80.2%. With regular cleaning, the media could be used for more than 50 cycles at high loading. Thus, Superose 12 HR 10/30 provides an alternative for the separation and purification of low molecular weight aromatic substances such as polyphenols, especially for water-soluble components that are popular in TCM preparations.

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