ISOLATION AND PURIFICATION OF PINELLIC ACID FROM *PINELLIA TERNATA*

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ABSTRACT

Pinellia ternata is a perennial herb belonging to the *Araceae* family. Pinellia ternata is a plant native to Japan, but also grows as a weed in certain areas of North America. It is commonly used as one of the main components in many decoctions in traditional Chinese medicine that has been used widely for thousands of years in China to prevent vomiting and for analgesic and sedative effects. *Pinellia ternata* is an active herbal component of the traditional Japanese herbal (Kampo) medicine, Sho-seiryu-to (SST) (Chinese name: Xiao-Qing-Long-Tang), and it has been reported to show oral adjuvant activity for nasally administered influenza hemagglutinin (HA) vaccine. The active compound that displayed adjuvant properties was identified as 9*S*, 12*S*, 13*S*-trihydroxy-10*E*-octadecenoic acid commonly known as Pinellic acid. This research was conducted with a goal to isolate and purify Pinellic Acid (9*S*, 12*S*, 13*S*-trihydroxy-10*E*-octadecenoic acid) from *Pinellia ternata* plant. *Pinellia ternata* was extracted with two types of solvent; water and methanol. The extracts were purified with Hydrophobic Interaction Chromatography with methanol as the polar eluent and Column Chromatography with the solvent system hexane-ethyl acetate. The pure Pinellic acid obtained was determined by Reversed phase High Performance Liquid Chromatography (RP-HPLC) using 70% methanol containing 0.01% acetic acid as the solvent system; and Infrared Spectroscopy. Highest % yield of Pinellic acid calculated was obtained from the sample extracted with methanol. High Performance Liquid Chromatography analysis for methanol-extract gave a clear single peak at 4.11 minutes at a flow rate of 1 ml/min. The compound obtained has displayed solvent selectivity towards methanol as opposed to water.

ABSTRAK

Pinellia ternata adalah herba yang tergolong dalam keluarga *Araceae*. *Pinellia ternata* berasal dari Negara Jepun, tetapi juga membiak secara liar di kawasan-kawasan tertentu di Amerika Utara. Herba ini biasanya digunakan sebagai salah satu komponen dalam rebusan air ubat tradisional Cina yang telah digunakan secara turun-temurun untuk mencegah muntah and ubat pelali. Pinellia ternate ialah komponen actif dalam ubat tradisional Jepun (Kampo), Sho-seiryu-to (SST) (Nama Cina : Xiao-Qing-Long-Tang) dan didapati boleh mengubati influenza hemagglutinin (HA). Kompaun aktif di dalam ubat ini dikenalpasti sebagai 9*S*, 12*S*, 13*S*-trihydroxy-10*E*-octadeenoic acid, lebih dikenali sebagai 'Pinellic acid'. Tujuan utama kajian ini dijalankan adalah untuk mengekstrak 'Pinellic acid' daripada Tuber Pinellia ternata. *Pinellia ternata* akan diekstrak menggunakan air dan methanol. Ektrak herba kemudiannya akan di lalukan melalui dua kaedah iaitu 'Hydrophobic Interaction Chromatography' dengan methanol sebagai eluen polar dan 'Column Chromatography'menggunakan hexane-ethyl acetate sebagai eluen. Kompaun 'Pinellic acid' was didapati akan dikenalpasti melalui 'Reverse-phased High Performance Liquid Chromatography (RP-HPLC)' menggunakan 70% methanol yang mengandungi 0.01% asid asetik sebagai eluen; dan 'Infrared Spectroscopy'. % kandungan Pinellic acid yang didapati adalah berasal dari sampel yang diekstrak dengan methanol. Analisa 'HPLC' menghasilkan satu puncak yang jelas bagi sampel ekstrak-methanol pada 4.11 minit dengan kadar aliran sistem 1 ml/min. Analisa HPLC menghasilkan satu puncak yang jelas bagi sampel ekstrak-methanol. Kompaun yang diperolehi daripada kajian ini telah menunjukkan selektiviti terhadap solvan methanol berbanding dengan air.

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$\tilde{\nu}$	Wavenumber
λ	Wavelength
RT	Retention time
cm^{-1}	Measurement of wavenumber
nm	Measurement of wavelength
mL	Volume

LIST OF ABBREVIATIONS

HIC	Hydrophobic interaction chromatography
CC	Column Chromatography
TLC	Thin Layer Chromatography
IEX	Ion exchange
GF	Gel Filtration
IUPAC	International Union of Pure and Applied Chemistry
HPLC	High Performance Liquid Chromatography
HPLC-UV	High Performance Liquid Chromatography – Ultra Violet
UV	Ultra Violet
IR	Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
LC-MS	Liquid Chromatography – Mass Spectrometer
MeOH	Methanol
PTFE	Polytetrafluoroethylene
XBXT	Xiao-Ban-Xia-Tang

CHAPTER 1

INTRODUCTION

The tuber of *Pinellia ternata* from the family Araceae is one of the main components in many decoctions in traditional Chinese medicine that has been applied since ancient times for anti-emetic, anti-tussive, sedative and anti-inflammatory purposes. (Marki et al., 1987) *Pinellia ternata* has been used widely for thousands of years in China to prevent vomiting and for analgesic and sedative effects. (Tsay et al., 1989) Phytochemicals from this plant that have been previously characterized include alkaloids (Zhao, 1990), volatile oils (Wang et al., 1995) and polysaccharides (Tomoda et al., 1994).

Pinellia ternata is an active herbal component of the traditional Japanese herbal (Kampo) medicine, Sho-seiryu-to (SST) (Chinese name: Xiao-Qing-Long-Tang), and it has been reported to show oral adjuvant activity for nasally administered influenza hemagglutinin (HA) vaccine. The active compound was identified as 9*S*, 12*S*, 13*S*-trihydroxy-10*E*-octadecenoic acid using infrared spectra, proton magnetic resonance, mass spectrometry, and circular dichroism, and named pinellic acid. The results of a study conducted by Nagai et al (2002) suggest that pinellic acid may provide a safe and potent oral adjuvant for nasal influenza HA vaccine as it showed no hemolytic activity. (Nagai et al, 2002)

Table 1.1 reflects some of the physical and chemical properties of Pinellic acid. The isolation and purification of pure Pinellic Acid in this study from *Pinellia ternata* will serve as a standard for future research and act as a vital marker for Quality Control in other herbs. A convergent synthetic route to produce pinellic acid has been developed via regioselective asymmetric dihydroxylation and

streoselective reduction. (Sunazuka et al, 2001) Pinellic acid is formed from the epoxy alcohol by action of epoxide hydrolase activity, which catalyzes epoxide opening at C-12. (Hamberg and Hamberg, 1996)

Table 1.1 : Physical and Chemical properties of Pinellic acid

Properties		Reference
Molecular Formula	$C_{18}H_{34}O_5$	(National Center for Biotechnology Information)
Molecular Weight	330.45956 g/mol	(National Center for Biotechnology Information)
Molar Volume:	307.545 cm3	(RSC, 2011)
Surface Tension:	45.699 dyne/cm	(RSC, 2011)
Flash Point:	289.331 °C	(RSC, 2011)
Boiling Point:	531.526 °C at 760 mmHg	(RSC, 2011)

1.1 Problem Statement

Kampo medicine (Chinese name : Xiao-Qing-Long-Tang) has been used clinically for treatment of cold syndromes. One component of the herbs in Kampo medicine is the tuber of *Pinellia ternata*. *Pinellia* Tuber which was found in the Guizhou Province of China has been found to contain Pinellic acid, an active compound that exhibits oral adjuvant activity for influenza vaccine.

At present, no research has been carried out in Malaysia on the isolation of Pinellic acid from the local *Pinellia ternata* species. The isolation of Pinellic acid in local *Pinellia ternata* may serve as a standard for chemical profiling of potential future researches.

1.2 Objectives

The overall goal of this study is to isolate and purify Pinellic Acid from *Pinellia ternata* plant.

The aims of this study are:

1. To extract *Pinellia ternata* tuber with two solvents of extraction; water and methanol.

2. To purify Pinellic acid (9*S*, 12*S*, 13*S*-trihydroxy-10*E*-octadecenoic acid) by fractionation with Hydrophobic Interaction Chromatography and Column Chromatography.

3. To identify and determine pure Pinellic acid compound.

4. To determine and compare % yield of Pinellic acid obtained from two methods of extraction.

1.3 Scope of Research

In the present study, Pinellic acid was extracted from *Pinellia ternata* Tuber, purified and identified. The details of the scope are as below :

(a) To extract *Pinellia* Tuber with Soxhlet apparatus, carried out with two extraction solvents separately, which are water and methanol.

(b) To purify extracts with Hydrophobic Interaction Chromatography and Silica Gel Column Chromatography.

(c) To identify Pinellic acid compound by running High Performance Liquid Chromatography and Infrared Spectroscopy.

(d) To compare solvent selectivity during extraction for both types of sample and to calculate % yield obtained.

1.4 Rationale and Significance

Rationale. The objective of the present study is to isolate and purify Pinellic Acid from powdered *Pinellia ternata*.

Significance. Isolation and purification of *Pinellia ternata* extract will produce a pure sample of Pinellic Acid. Pinellic acid will be identified preliminarily through High Performance Liquid Chromatography and Infrared Spectroscopy.

CHAPTER 2

LITERATURE REVIEW

2.1 Pinellia Ternata

Pinellia ternata (Chinese name Ban Xia), a perennial grass belonging to Araceae, is an important Chinese medicinal herb that has been used in clinical practice for over 2000 years. Table 2.1 shows the classifications of *Pinellia ternata* plant by the United States Department of Agriculture (USDA, 2011). *Pinellia ternata* (Figure 2.1) is one of the weeds in nonirrigated farm land, commonly seen in grassland, uncultivated land, corn fields, and under sparce woods. The species is also distributed in Japan and the Korean peninsular. (Bajaj, 1995) Many of these products are used for morning sickness. *Pinellia ternata*, along with ginger, is an ingredient in the herbal formulation Xiao-Ban-Xia-Tang (XBXT), which is used to prevent vomiting. However, since April 2004, Pinellia ternata has been banned in the US because it contains chemicals called ephedrine alkaloids. These chemicals might cause serious side effects such as heart attack, stroke, or seizures. (Ok et al, 2009)

Figure 2.1 : Wild *Pinellia ternata* plant.

Table 2.1 : Classifications of *Pinellia ternata* plant

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Liliopsida* – Monocotyledons
Subclass	*Arecidae*
Order	*Arales*
Family	*Araceae* – Arum family
Genus	*Pinellia* Ten. – pinellia
Species	*Pinellia ternata* (Thunb.) Makino ex Breitenbach – crowdipper

United States Department of Agriculture (USDA, 2011)

2.2 Pinellic Acid

Pinellic acid (Figure 2.2) and other trihydroxyoctadecenoates are produced in plants during wounding and infection by fungal pathogens (Kato et al, 1991). Interestingly, such trihydroxy oxylipins inhibit growth of fungi and germination of spores (Masui et al, 1989 and Walters, 2006) and may play a role in plants' defense towards pathogenic fungi. The compound is also present in beer (Esterbauer and Schauenstein, 1977) and may contribute to the bitter taste of this beverage. (Baur and Grosch, 1977)

Pinellic acid has been found to be a potentially useful oral adjuvant extracted from the tuber of *Pinellia ternata* (Nagai et al, 2002) used in conjunction with intranasal inoculation of influenza HA vaccines. Among this series of isomers, 9*S*, 12*S*, 13*S* compound has the most potent adjuvant activity. According to a study conducted by Shirahata et al (2002), the adjuvant activity of (+)-1, the enantiomer of natural pinellic acid, was weaker than that of the natural one. However, adjuvant activity of pinellic acid (−)-1 from a natural source was lower than that of the synthetic one. (Shirata et al, 2002)

In a study conducted by Miura and Kuwahara (2009), a new enantioselective synthesis of Pinellic acid, a trihydroxy unsaturated fatty acid exhibiting oral adjuvant activity for nasally administered influenza vaccine, has been accomplished using a cross-metathesis reaction between two terminal olefin intermediates as the key step. This synthesis is the shortest to date, furnishing Pinellic acid in 17% overall yield via only seven steps from a readily available known dihydroxy ester. (Miura and Kuwahara, 2009)

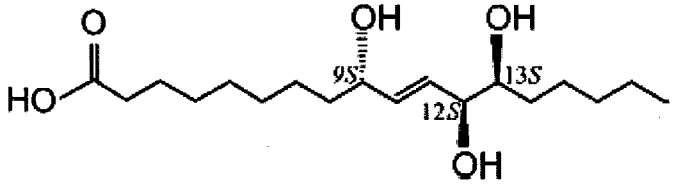

Figure 2.2 : Structure of Pinellic acid (9S, 12S, 13S-trihydroxy-10E-octadecenoic acid)

2.3 Freeze Drying

Freeze-drying is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport. Freeze-drying freezes the material, and then reduces the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase. Vacuum Freeze Drying is noninvasive and produces no chemical damage.

If a freeze-dried substance is sealed to prevent the reabsorption of moisture, the substance may be stored at room temperature without refrigeration, and be protected against spoilage for many years. Preservation is possible because the greatly reduced water content inhibits the action of microorganisms and enzymes that would normally spoil or degrade the substance.

Freeze-drying also causes less damage to the substance than other dehydration methods using higher temperatures. Freeze-drying does not usually cause shrinkage or toughening of the material being dried. In addition, flavours, smells and nutritional content generally remain unchanged, making the process popular for preserving food. However, water is not the only chemical capable of sublimation, and the loss of other volatile compounds such as acetic acid (vinegar) and alcohols can yield undesirable results.

Freeze-dried products can be rehydrated much more quickly and easily because the process leaves microscopic pores. The pores are created by the ice crystals that sublimate, leaving gaps or pores in their place. This is especially important when it comes to pharmaceutical uses. Freeze-drying can also be used to increase the shelf life of some pharmaceuticals for many years. (Snowman, 1988)

2.4 Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography (HIC) is a liquid chromatography technique that separates biomolecules according to hydrophobicity. Although most hydrophobic amino acids are buried in the interior of globular proteins, some of them are exposed on the protein surface. These can interact with hydrophobic ligands on the HIC gel. The amount of exposed hydrophobic amino acids differs between proteins and so does the ability of proteins to interact with HIC gels. HIC is complementary to ion exchange (IEX) and gel filtration (GF) and high overall resolutions are obtained when HIC is combined with other Liquid Chromatography techniques. High salt necessary for binding may sometimes cause precipitation of some sample components including the target protein.

HIC (Figure 2.3) media contain ligands that can combine with hydrophobic surfaces of proteins. In pure water, this hydrophobic effect is too weak to cause interaction between either ligand and proteins, or between the proteins themselves. However, certain salts enhance hydrophobic interactions and adding such salts brings about adsorption to HIC media. The order of salts for which hydrophobic interaction is strengthened are :

Na_2SO_4 > K_2SO_4 > $(NH_4)_2SO_4$ > NaCl > NH_4Cl > NaBr > NaSCN

Ammonium sulfate is the salt commonly used to control adsorption in HIC. The sample is applied and adsorbed at high salt concentrations (~ 1M). To bring about selective desorption, the salt concentration is then lowered gradually and the sample components elute in order of hydrophobicity.

In 'hydrophobic interaction chromatography', a form of multivalent interaction chromatography, the solutes (native proteins) are 'adsorbed' and separated on a stationary solid phase (i.e. a two-dimensional system) carrying immobilized hydrophobic groups. One of the prerequisites for an optimal chromatographic separation of proteins by hydrophobic interactions is an extremely hydrophilic, minimally interactive stationary phase. (Jennissen, 2005)

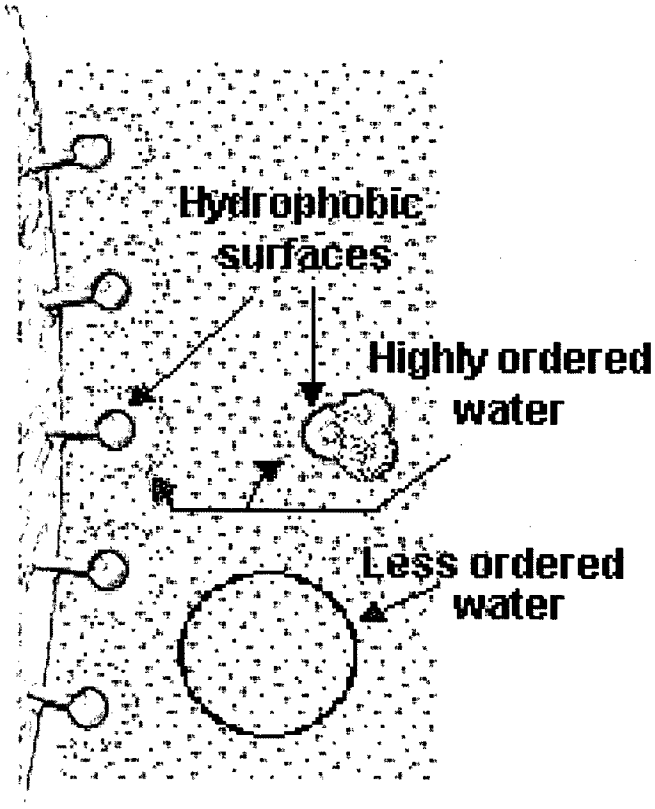

Figure 2.3 : HIC deals with the relation between water shells around hydrophobic surfaces, with bulk water clusters and salts enhancing hydrophobic interaction.

2.5 Column Chromatography

Column Chromatography (CC) is a common and useful separation technique in organic hemistry. Column chromatography can be used on both a large and small scale. The applications of this technique are wide reaching and cross many disciplines including biology, biochemistry, microbiology and medicine. Many common antibiotics are purified by column chromatography. Column chromatography separates and collects the compounds individually. As with TLC, alumina and silica are the two most popular stationary phases in column chromatography. For these common phases, the partitioning works in an analogous manner. The more polar sample will be retained on the stationary phase longer. Thus the least polar compound will elute from the column first, followed by each compound in order of increasing polarity.

Stationary phases for CC can come in a variety of sizes, activities, acidic and basic variations for both alumina and silica. Normally, a separation will begin by using nonpolar or low polarity solvent, allowing the compounds to adsorb to the stationary phase, then slowly switching the polarity of the solvent to desorb the compounds and allow them to travel with the mobile phase. The polarity of the solvents should be changed gradually.

Columns can be as thin as a pencil to a diameter of several feet in industrial processes. They can separate milligram to kilogram quantities of materials. There are several acceptable methods when packing a column. These include dry packing and the slurry method. The slurry method normally achieves the best packing results.

Dry packing is the method of choice for a microscale column. The column is first filled with a nonpolar solvent. Powdered alumina or silica is then added slowly while gently tapping the side of the column with a pencil. The solid should "float" to the bottom of the column. Try to pack the column as evenly as possible; cracks, air bubbles, and channels will lead to a poor separation. For the second dry pack method, the stationary phase is deposited in the column before the solvent. In this

case the column is filled to the intended height with the stationary phase and then slowly add the nonpolar solvent. The solvent should be added slowly as to avoid uneven channeling. This method is typically used with alumina only, since silica gel expands and does not pack well with this dry method.

The slurry method is often used for macroscale separations. Combine the solid stationary phase with a small amount of nonpolar solvent in a beaker. The two will be mixed until a consistent paste is formed, but is still capable of flowing. Pour this homogeneous mixture into the column as carefully as possible using a spatula to scrape out the solid as you pour the liquid. The slurry method normally gives the best column packing, but is also a more difficult technique to master. Whether the dry or slurry method is chosen, the most important aspect of packing the column is creating an evenly distributed and packed stationary phase. As mentioned, cracks, air bubbles and channeling will lead to a poor separation.

Once the column is loaded, the stopcock is opened and the solvent level is allowed to drop to the top of the packing, but the solvent layer should not go below this point.

Normally, a minimum amount of a polar solvent, 5-10 drops, is used to dissolve the mixture. The solution is then carefully added to the top of the column using a pipette without disrupting the flat top surface of the column. A thin horizontal band of sample is best for an optimal separation. After the sample is loaded, a small layer of white sand is added to the top of the column. This will help to keep the top of the column level when adding solvent eluent. Once the mixture is added and the protective layer of sand is in place, continuously add the solvent eluent while collecting small fractions at the bottom of the column. A pipette is used to add the first bit of solvent on top of the packing, sample, and sand will minimize disturbance of the column and diluting the sample.

Collecting small fractions (1-3 mL) is important in column separation. Fractions that are too small can always be pooled together; however, if the collected fractions are too large, more than one compound may be present in any particular

fractionSince column chromatography is time consuming, collecting large fractions is discouraged. Once it is believed all the materials have been removed from the column, combine the like or same fractions and evaporate the solvent. The pure separated compound will be left behind. Recrystallization may be used to further purify a solid product. However, on a milligram scale, there is usually not enough material to do this. (David, 2001)

2.6 Reverse-phase HPLC

HPLC (Figure 2.4) is a popular method for the analysis of herbal medicines because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. In general, HPLC can be used to analyze almost all the compounds in the herbal medicines. Thus, over the past decades, HPLC has received the most extensive application in the analysis of herbal medicines. Reversed-phase (RP) columns may be the most popular columns used in the analytical separation of herbal medicines. On the other hand, the advantages of HPLC lie in its versatility for the analysis of the chemical compounds in herbal medicines. With the additional UV spectral information, the qualitative analysis of complex samples in herbal medicines turns out to be much easier than before. For instance, checking peak purity and comparing with the available standard spectrum of the known compound to the one in the investigated sample. (Springfield et al, 2004)

High Performance Liquid Chromatography – Diod Array Detector (HPLC-DAD) is being increasingly utilized for the screening of drugs, vitamins and natural products and has also been applied to the identification of poisoning by traditional medicines. In HPLC, qualitative solute identification can be achieved via comparison of retention data. (Springfield et al, 2004)

Reversed phase HPLC has a non-polar stationary phase and an aqueous, moderately polar mobile phase (solvent). Reverse-phase HPLC is the separation method of choice for most pharmaceutical compounds, both hydrophilic and hydrophobic, due to the stable, reproducible nature of the HPLC columns, the largely aqueous composition of the mobile phase and the relative ease of reproducing the

methods in a variety of laboratories. Reversed Phase Chromatography results from the adsorption of hydrophobic molecules onto a hydrophobic solid support in a polar mobile phase. Decreasing the mobile phase polarity by using organic solvents reduces the hydrophobic interaction between the solute and the solid support resulting in de-sorption. The more hydrophobic the molecule the more avidly it will adsorb onto the solid support. This requires a higher concentration of organic solvent to promote de-sorption. (Montgomery et al, 2000)

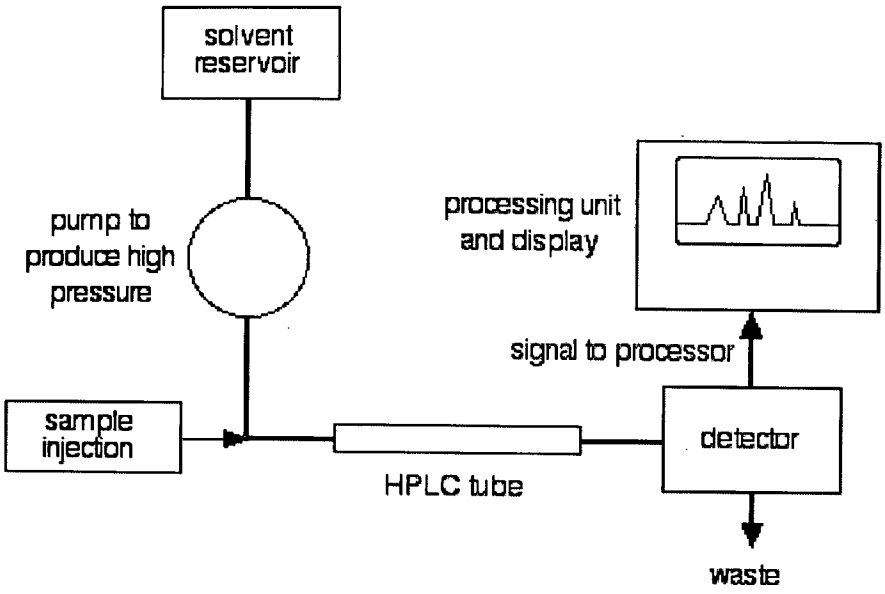

Figure 2.4 : Flow scheme for HPLC