CHAPTER 4

Electrocoagulation of Black Bean and a Small Scale Electrocoagulation

4.1 Introduction

The common bean (*Phaseolus vulgaris* L.) is the world's second most important bean after soybeans and is one of the basic foods of the indigenous population in South America. The characteristic intense red anthocyanin pigments in the skin of black beans make them an attractive potential source for natural food colors. Anthocyanin pigments may be used for coloring foodstuffs and snack food, beverages, pharmaceutical, and cosmetic products and for coloring textiles, paper, and leathers.

The study of Takeoka, G.R. and coworkers (1997)[48] reported to the determination of the anthocyanin content in black beans by using a combination of analytical methods such as preparative HPLC, TLC, GC, UV-vis spectroscopy, MS, NMR spectroscopy. The results showed that the total anthocyanin content of black beans was 213 $\pm$ 2 mg/100 g of beans, moisture content was 10.04 $\pm$ 02%. The anthocyanins in black beans appear to occur exclusively in the seed coat, which comprised 8.98 $\pm$ 0.17% of the whole bean. Thus, the anthocyanin content in the seed coat of black beans is about 2.37 g/100 g of seed coat or 2.37%. The HPLC chromatogram of black bean extract detected in the visible spectral region (520 nm) revealed three major anthocyanins; delphinidin 3-glucoside (56%), petunidin 3-glucoside (26%), and malvidin 3-glucoside (18%).

In this study, electrocoagulation technique was applied to isolate coloring matter in black bean. The recovery coagulum was analyzed by HPLC compared with unelecctrolysis crude extract.

4.2 Experiment

4.2.1 Plant material

Black bean sample was purchased from a Chiang Mai's local market.

Picture 4.1 Black bean

4.2.2 Instrumental and apparatus

4.2.2.1 Electrocoagulation experiment

(1) UV/VIS spectrophotometer, model Genesys 10 spectrophotometer, Thermo scientific, USA.

(2) DC power supplier, model GPR-1810 HD and GPS-3030D; Good Will Instrument Co.Ltd., Taiwan.

(3) Vacuum rotary evaporator, model rotavapor-R; Buchi Vacuum pump, model PC-1; VacUUbrand, Imed, USA.

(4) pH meter, model pH; Precisa, Merck, Germany.

(5) TLC plates (aluminium), silica gel 60 F_{254}; Merck, Germany.

(6) Aluminium plates (dimension 15 x 3.5-4 x 0.05cm)

4.2.2.2 HPLC separation

(1) High Performance Liquid Chromatography (HPLC) with UV-970 intelligent UV2Vis detector, Jasco, Japan

(2) HPLC pump, PU-980 intelligent, Jasco, Japan

(3) Syringe Driven Filter unit; low protein binding hydrophilic LCR 0.20 μm ; Millex - LG

(4) (PTFE) membrane (4 mm) for clarification of aqueous and organic solutions.

4.2.3 Chemicals

(1) *n*-Butanol $C_4H_{10}O$ (Analytical Reagent grade); Fisher Scientific UK Ltd., UK.

(2) Hydrochloric acid solution HCl (37%); Carlo Erba Reagent Co., Ronando, MI, Italy.

(3) Ethanol C_2H_5OH (absolute); E. Merck Darmstadt, Germany.

(4) Sodium chloride NaCl (99.9%, AR grade); Ajax Chemical Co. Sydney, Australia.

For HPLC experimental

(1) Acetronitrile CH_3CN (for GC); Nacali tesque, Japan.

(2) Trifluoroacetic acid TFA; CF_3COOH (Analytical Reagent grade); Nacali tesque, Japan.

(3) Methanol CH_3OH (for GC); Nacali tesque, Japan.

(4) Ethanol C_2H_5OH (Analytical Reagent grade), Wako, Japan.

(5) 1-Butanol $CH_3(CH_2)_2CH_2OH$ (for GC); Nacali tesque, Japan.

(6) Milli Q water

4.2.4 Procedure

Electrocoagulation of black bean

(a-1) Pigments extraction of black bean

Aqueous ethanol (85%, 500 mL) with 3% v/v trifluoroacetic acid solution was added to 50.13 g of dry grain black bean and stirred overnight at room temperature. After filtration the filtrate was made up to a volume of 500 mL and divided into 2 sets: 250 mL each. The first 250 mL of extraction solution was evaporated and dried in vacuum. The red glutinous compound was obtained (3.29 g; 13.1% yield).

(a-2) Electrocoagulation

Another 250 mL of sample solution from experiment (a-1) was placed in a 400 mL beaker then 0.52 g of sodium chloride was added. A pair of 15 x 4 x 0.05 cm aluminum electrodes space 3 cm apart and dipped 5.5 cm into a magnetically-stirred solution. Direct current (0.4-2.0 A, 26.2-31.5 V) was passed through the mixture for 2 hr. Every 15 min during a period of electrolysis, a 4 mL sample of the solution was withdrawn, centrifuged and taken for an absorbance measurement at an appropriate wavelength (535 nm) and then plotted with electrocoagulation time.

(a-3) EC filtrate recovered compound preparation

Aqueous ethanol (80%, 250 mL) with 3% v/v trifluoroacetic acid solution was added to 25.01 g of dry grain black bean and stirred overnight at room temperature. After filtration the filtrate was made up to a volume of 250 mL.

Sodium chloride (0.50 g) was added into 250 mL of sample solution. The solution was placed in a 400 mL beaker, direct current (0.5 A, 13.5 V) was then passed through

the solution via the two aluminium plates electrodes (15 x 4 x 0.05 cm spaced 3 cm apart and dipped 5.5 cm deep into a magnetically-stirred solution) for 2 hr.

After electrocoagulation the mixture was filtered and the filtrate was evaporated. The residue was dissolved in absolute ethanol and the remaining undissolved inorganic matter was then filtered off from the solution. Small amount of concentrate TFA was added to the solution until bright red color was appeared. The solution was evaporated to dryness in vacuum. After extraction the solution was obtained by filtration then evaporated and dried in vacuum. The dark red solid residue was obtained (3.8 g ; 15.2 % yield)

Extraction and electrocoagulation of black bean was repeated in the same steps as above with a DC 1.50 A current (26.2 V) for 2 hr. After coagulation, the brown solid (3.2 g; 2.7% yield) was recovered from the filtrate.

(b) HPLC of crude extract and recovered compounds from EC filtrate of black bean

(b-1) Solubility test

Water : acetronitile solvent system was prepared, the portion varying from 100% to 0 % v/v of water. The solvent (2 mL) was placed in test tube and a small quantity of sample was added. After shaking for 1 min the tested sample solution was investigated, solubility and color were recorded. Color and solubility of black bean sample are shown in Picture 4.3 and Table 4.1

(b-2) HPLC analysis

Sample preparation

Three samples were prepared for HPLC analysis: the first one was crude extraction of black bean (85% aqueous ethanolic solution with 3% TFA; sticky red compound); next was a crude extraction of black bean with EC process (with low current 0.5 A which was a solid dark purple compound), and the last one was a crude extraction of black bean with EC process (with high current 1.5 A ; solid pale brown compound).

Each of the extracts (0.01 g) was dissolved in 1 mL of 50:50 (v/v) acetronitrile : water. The sample solution was filtered via a syringe driven filter unit to clean up and then applied to HPLC column.

HPLC separation condition 1 [49]

In this experiment the three samples were analyzed at the 535 nm detection wavelength. The column needed to be equilibrated with the first solvent for 30 min before starting to separate.

Column: Devolosil ODS-5

Mobile Phase: 0.1% TFA (A) and acetronitile (B)

Gradient condition: 10-15% B for 20 min,

15-18% B for 10 min

18-35% B 20 min.

Flow rate: 0.5 mL/min

Detector: UV-Vis 535 nm

HPLC separation condition 2

One step gradient separation of black bean crude extract from EC process (0.5 A).

Column: Devolosil ODS-5

Mobile Phase: 0.1% TFA (A) and acetronitile (B)

Gradient condition: 90-65% A for 75 min

Run time: 90 min

Flow rate: 0.5 mL/min

Detector: UV-Vis 520 nm

HPLC separation condition 3

In this experiment the same condition as in experiment 2 was used and runtime was expanded from 90 to 120 min.

Column: Devolosil ODS-5

Mobile Phase: 0.1% TFA (A) and acetronitile (B)

Gradient condition: 90-65% B for 75 min

Run time: 120 min

Flow rate: 0.5 mL/min

Detector: UV-Vis 520 nm

(c) Small scale electrocoagulation of crude black bean

Black bean crude extract (82.5 mg) was dissolved in 20 mL 85% aqueous ethanol. Sodium chloride (0.04 g) was added. The sample solution was placed in a 25 mL bottle and direct current 20 mA (19-24 V) was passed through the solution via two aluminium electrodes. At every minute during a 15-min period of electrolysis, a 200 µL aliquot sample of the solution was drawn and the supernatant solution was taken for HPLC analysis.

HPLC condition:

Column:	Devolosil ODS-5
Flow rate:	0.5 mL/min
Volume of injection:	10 µL
Solvent	A: 0.1% TFA B: acetonitrile
Gradient	10-15 %B for 20 mins, 15-18% B for 10 mins
	18-35% B for 20 mins
Detection	UV-Vis 535 nm

4.3 Results and discussion

Electrocoagulation of black bean

Plot of absorbance and electrolysis time during electrocoagulation process is shown in Figure 4.1, and fractions taken are shown in Picture 4.2.

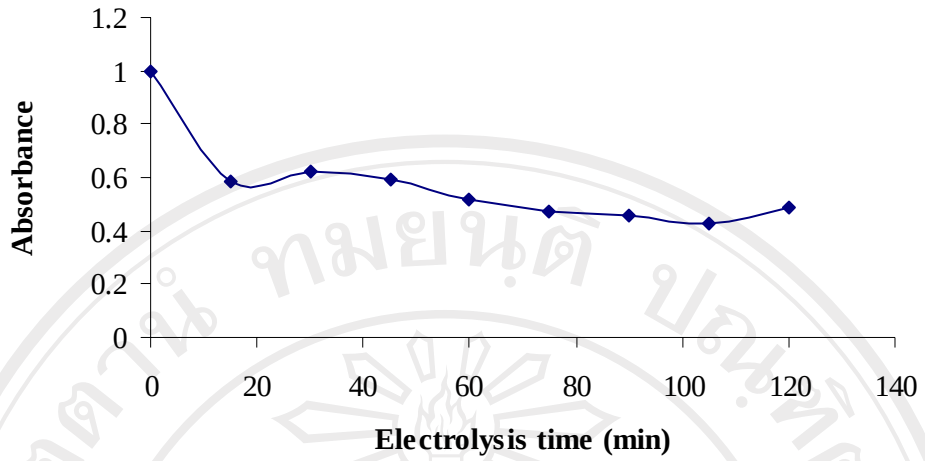


Figure 4.1 Plot of adsorbance versus electrocoagulation time during 2 hr of EC process of black bean (85% aqueous ethanol with 3% v/v trifluoroacetic acid).

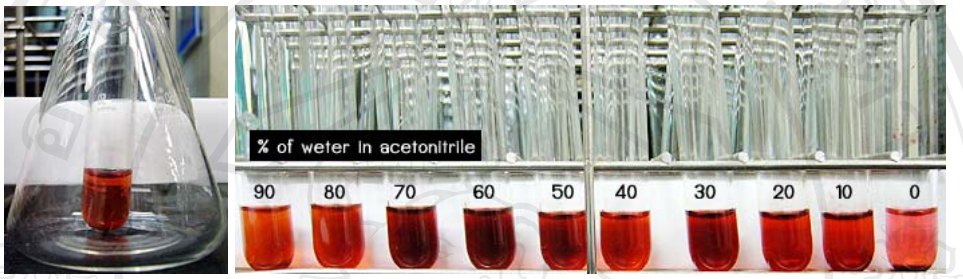
Picture 4.2 Black bean EC fractions taken every 15 min during 2 hr of EC process.

The result shows that black bean solution could not coagulated under studied condition.

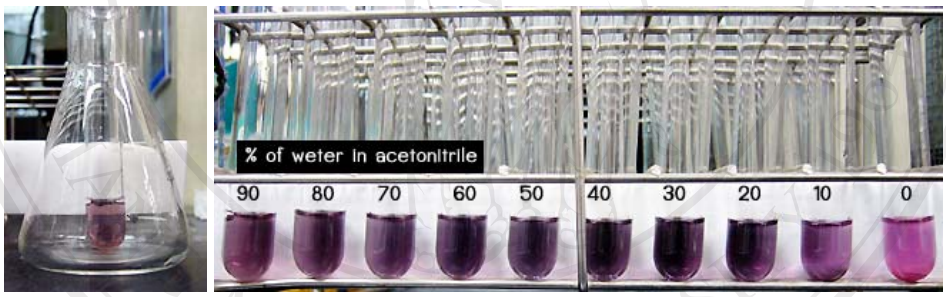
In the next section, the same experiment was repeated, electrocoagulation of black bean solution at the two different voltages was studied. Recovered compounds were analyzed by HPLC compared with the uncoagulated crude extract.

HPLC analysis of sample

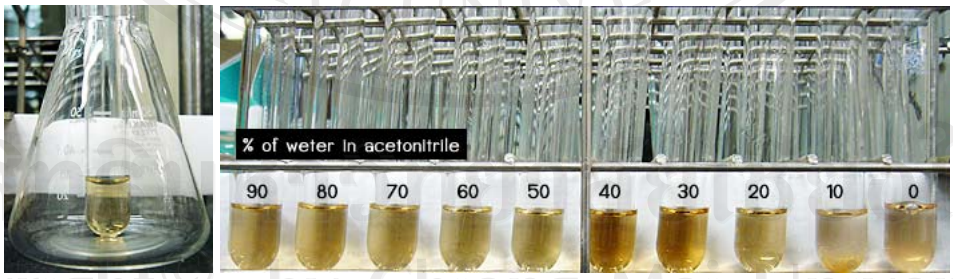
The solubility of crude extract and EC recovered compounds of black bean were tested. The appropriate solvent system was used for sample preparation for HPLC analysis. Solubility of sample are shown in Table 4.1 and the color obtained are shown in Picture 4.1



(a)



(b)



(c)

Picture 4.3 Solubility test of black bean crude extract (a), recovered compound from filtrate after EC process, 0.5 A (b) and 1.5 A (c) current. On the left is an aqueous solution of the tested sample (100% water), the right is a set of water:acetonitrile solvent system varying from 90 to 0 % water (100% acetonitrile).

Table 4.1 Solubility test of black bean samples.

Sample	%water	%acetronitile	Crude extract (red)	EC process 0.5 A (purple)	EC process 2 A (brown)
1	100	0	+	+++	+++
2	90	10	+++	+++	+++
3	80	20	+++	+++	+++
4	70	30	+++	+++	+++
5	60	40	+++	+++	++++
6	50	50	++++	++++	++++
7	40	60	++++	++++	++++
8	30	70	++++	++++	++++
9	20	80	+++	++++	++++
10	10	90	+++	++	++
11	0	100	+	+	++

Note + some undissolve solid residue with pale color.

+ + small quantity of solid residue with clear color.

+ + + small quantity of solid residue with bright clear color.

+ + + + completely dissolve with a strong color.

From the results above, 50% acetonitrile in water was chosen for sample preparation.

HPLC chromatograms

Three samples of black bean were analyzed by HPLC technique. HPLC chromatograms were shown in Figure 4.2

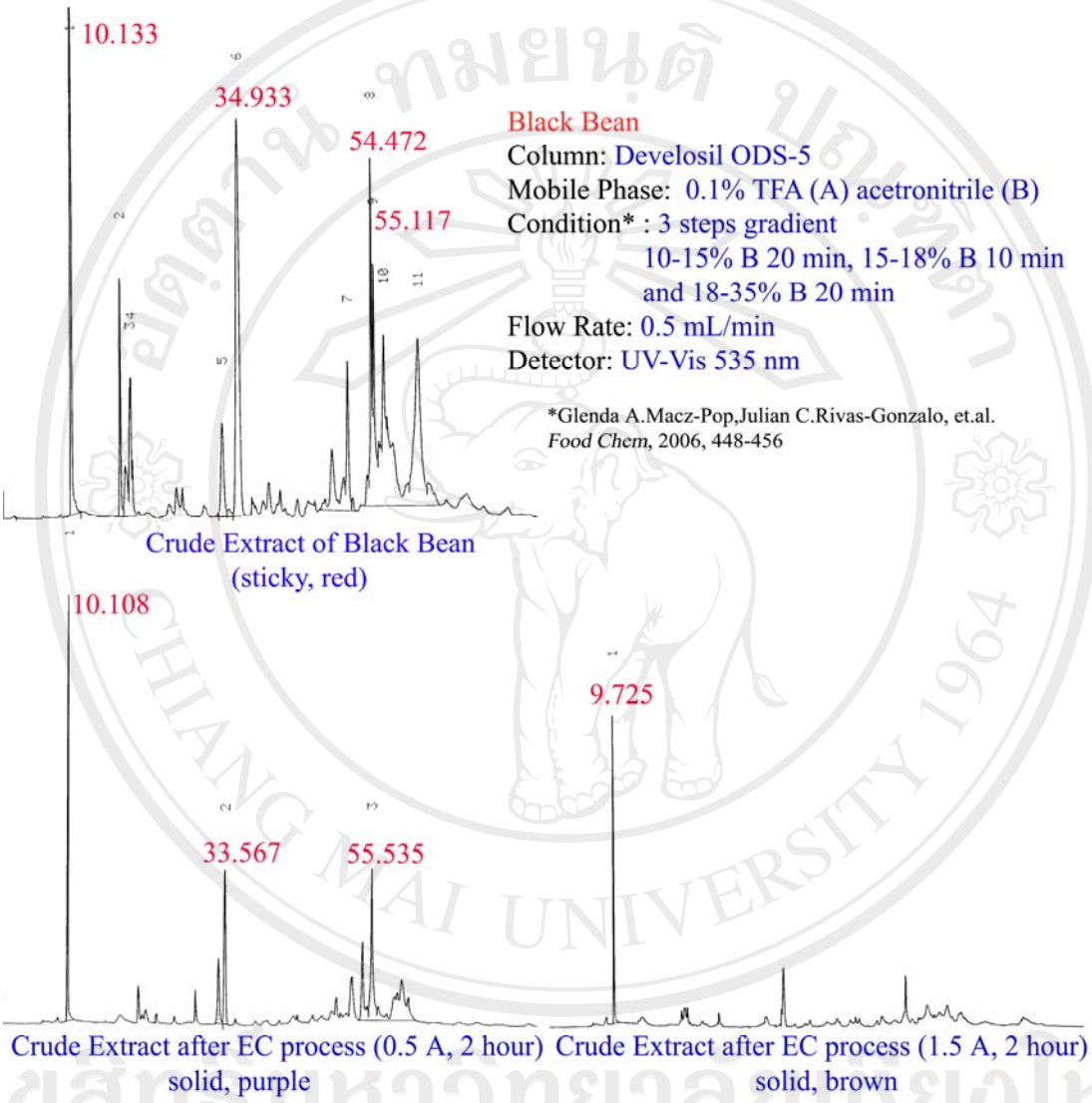


Figure 4.2 HPLC analysis of black bean crude extract, 0.5 A and 1.5 A recovered compound.

The result shows that the number of peaks appearing for each fraction were related to their color. The bright sticky red crude extract shows four main peaks at 10.133, 34.933, 54.472 and 55.147 min. Three main peaks at 10.108, 33.567 and 55.535 min were obtained from the low current EC fraction (purple compound) and only one peak at 9.725 min was obtained from the high current EC sample (brown compound).

Other HPLC separation conditions were tried to analyze the sample. As the one step gradient (second condition) was used and the third condition in which analyzed time was expand, HPLC chromatograms are abtained as shown in Figures 4.3 and 4.4 respectively.

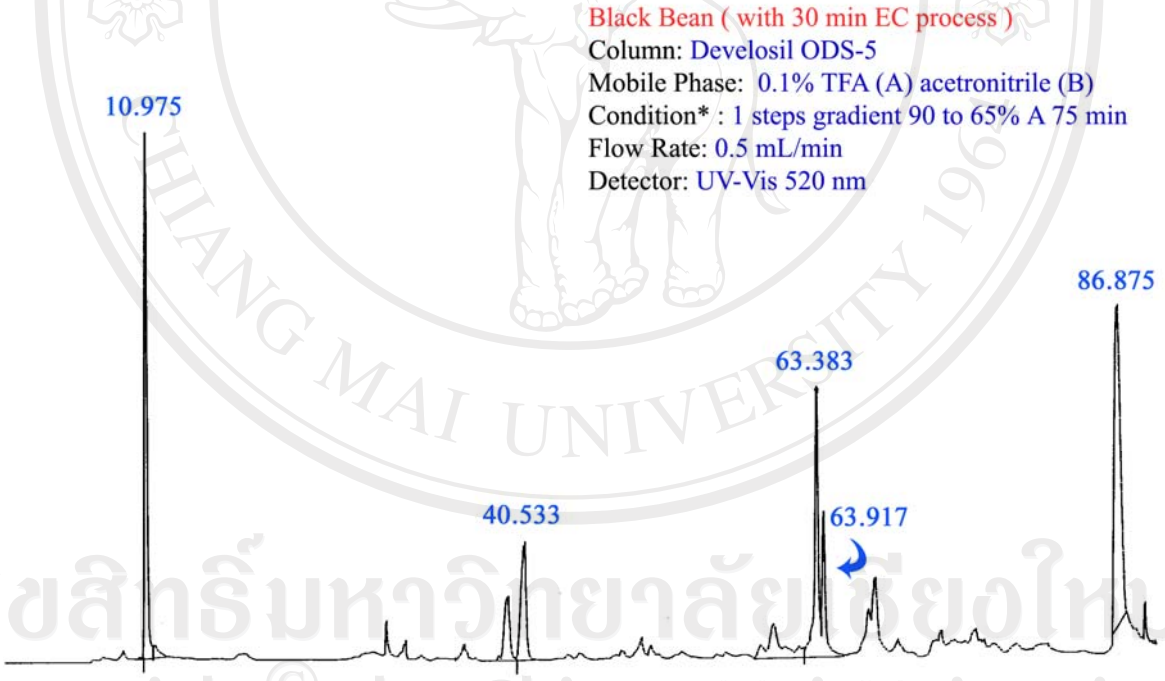


Figure 4.3 HPLC chromatogram of black bean obtained from HPLC second condition.

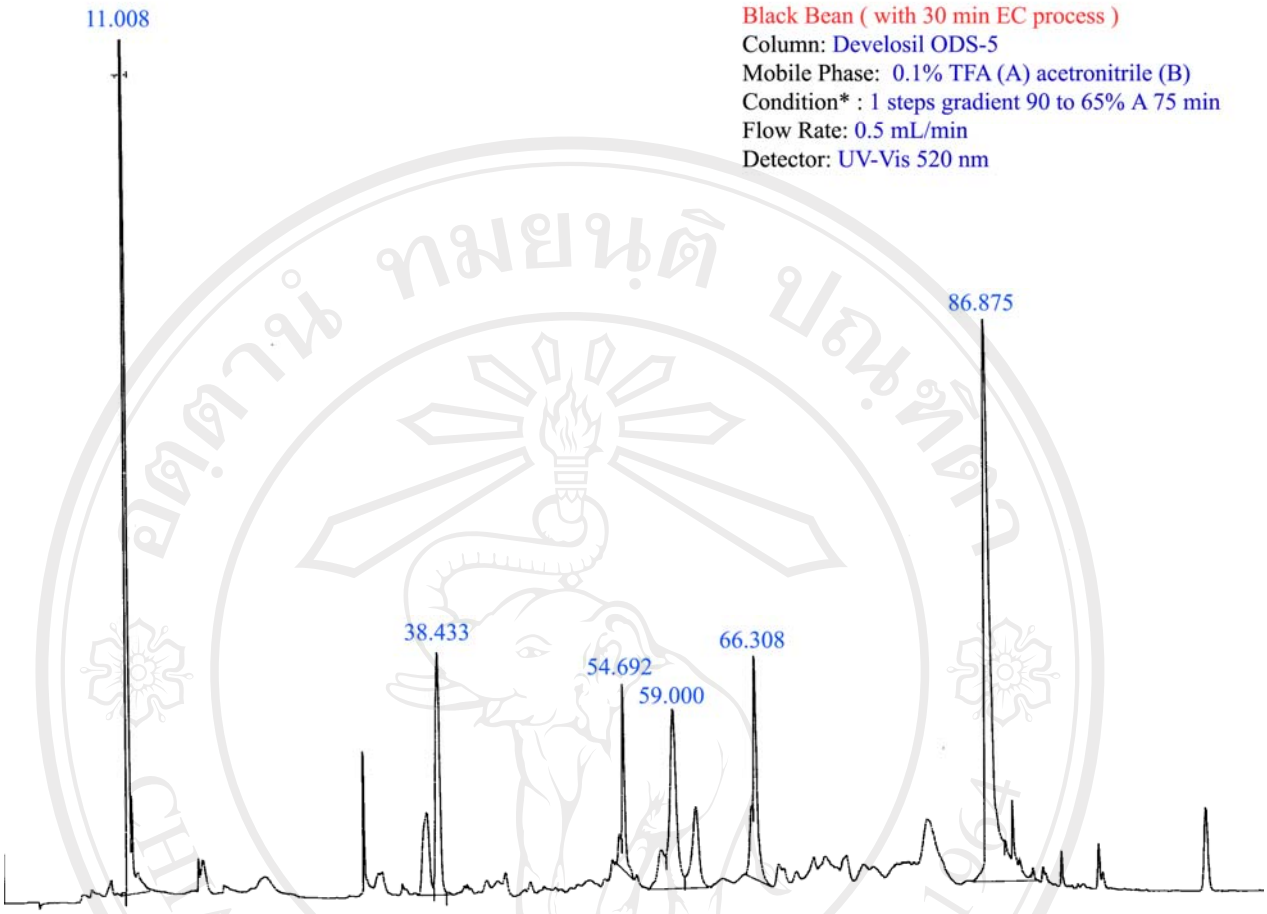


Figure 4.4 HPLC chromatogram of black bean obtained from HPLC condition 3

As illustrated in the chromatogram, the crude extract of black bean sample shows the best separation in the third HPLC condition. However, this condition was not appropriate for the recovered fraction. After several tries the recovered fraction still showed low signals and was not separated.

In the previous study electrocoagulation with at least 250 mL of starting solution were attempt. This scale was set up and monitored by measurement of the sample's UV-Vis absorption. When HPLC was used as the monitoring system during EC process, the large scale process was not necessary.

In this study a 25-mL bottle was used as an EC chamber, two rolled aluminum foils were used as electrodes (as shown in the Picture 4.4), and a 20 mA current was used for electrocoagulation.

Picture 4.4 Small scale electrocoagulation.

After EC process the supernatant part was taken for HPLC analysis. However, the resulting chromatogram showed a low signal even when the concentration of starting solution was increased.

4.4 Conclusions

The coloring matter in black bean could not be coagulated under the studied electrocoagulation conditions. However, the EC filtrate can be recovered in a dry solid form. It can be seen that electrocoagulation with low current gave a brighter and stronger color recovered compounds than the high one.

HPLC technique was used for the separation of the coloring matter from black bean. The results show that devolosil ODS-5 column eluted by gradient condition of 90 to 65% acetronitle in 0.1% TFA for 75 min is an appropriate condition for separation of black bean crude extract. A small-scale electrocoagulation was set up to reduce the consumption of the substance used.

Overall Concluding Remarks

In this study, it has been determined that a variety of structurally different quinones were susceptible to electrocoagulation in different degrees depending on the number and position of their phenolic hydroxyl groups. Those quinones that are well coagulated can be recovered by a simple procedure. On the other hand, there is no selectivity for studied flavonoids by EC. This finding was applied to the isolation of some quinones and flavonoids from natural source. The data generated through this work may be used as a basis for studying the separation of compounds from a mixture of various quinones and flavonoids by EC.

It has been demonstrated in a systematic manner that EC in aqueous alcoholic solutions of some important plant pigments, eg. tannins and flavonoids (morin), the decrease in the percentage of water in the solvent has some small negative effect on the degree and efficiency of their coagulation compared with that observed in 100% aqueous solution. However, even with this unfavorable effect being present, electrocoagulation is still more efficient in removing these organic matrix substances than the conventional method of solvent extraction.

When EC was applied to separate the coloring matter from nine natural sources, the coloring matter from black glutinous rice and betel nut can be electrolysed and recovered in a dry-solid form. In addition, this process proved to be an efficient method for removing coloring matter from seedlac. In case of black bean the coloring matter could not be coagulated but was recovered in a bright shade color and dried-solid form from the filtrate after a low voltage current EC. This EC process may have application for an economic isolation of natural dyes on commercial scale. However, the coloring matter obtained by this method may not be suitable for use as a food additive due to the

probable structure change of the dyes and an amount of aluminium remaining. More study should be carried out with regard to this.

Attempts were made in using EC to separate coloring matter from jackfruit wood, roselle, beet root, gardenia and turmeric but the results were not quite satisfactory. Separation tries of morin from jackfruit wood, curcumin from turmeric, and crocin from gardenia showed that the recovered compounds were not identical to the authentic samples. The changed structures of the compounds still cannot be characterised. In this case, further detailed study by nmr spectra and mass spectra is suggested.

A small scale EC was attempted, this experiment set up was useful in decreasing the studied substance employed. However, it required a highly efficient monitoring system such as HPLC to monitor the various chemical components present during EC process.