COST ACTION FP1203

EUROPEAN NON-WOOD FOREST PRODUCTS (NWFPs) NETWORK

ENRICHMENT OF THE GLYCYRRHIZIC ACID FROM LICORICE ROOTS (*Glycyrrhiza glabra* L.) BY ISOELECTRIC FOCUSED ADSORPTIVE BUBBLE CHROMATOGRAPHY

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I. INTRODUCTION

a. Licorice Roots (*Glycyrrhiza glabra* L.)

Plants are a wide source of products used in the effective chemicals industry (pharmacy, flavors, fragrances, and pesticides). Secondary metabolism especially isolated and extracted from higher plants are valued from several thousand euro for per gram in the specialist chemicals industry.

Figure 1. General imaging about *Glycyrrhiza glabra* L. (URL 2, URL 3).

Glycyrrhiza glabra (Licorice) is a ligneous perennial shrub growing in Mediterranean region and Asia widespread in Turkey, Italy, Spain, Russia, Syria, Iran, China, and Israel. (Asada, Li, & Yoshikawa, 2000 ; Casulli & Ippolito, 1995). The plant, having multi-year production-cycle, has blue, violet flowers (Mastro et al., 1993). Liquorice roots are cylindrical in shape having a diameter of 0.5±2.5 cm and length of 15-20 cm (Marzi et al., 1993). In Figure 1 and 2, it is showed Distribution and general imaging of *Glycyrrhiza glabra* in Turkey such as Gaziantep, Iğdır, Kars, Siirt, Bitlis, Diyarbakir, Kahramanmaraş, Muş, Samsun, Şanlıurfa.

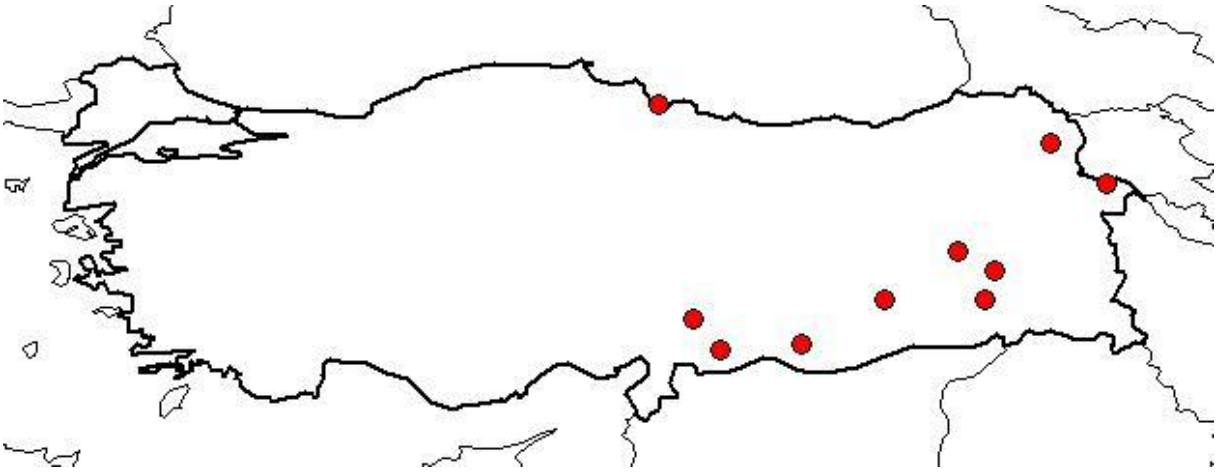

Figure 2. Distribution of *Glycyrrhiza glabra* in Turkey (URL 1)

b. Significant and Use Liquorice Roots

Glycyrrhiza glabra (Licorice) is a favourable herb used in food and medicinal remedies for thousands of years. This herb has long been valued as a demulcent (soothing, coating agent), to relieve respiratory ailments (such as allergies, bronchitis, cold, sore throats and tuberculosis), tomach burn including heartburn from reflux or any other cause, gastritis, inflammatory disorders, skin diseases and liver problems (Xu et al., 2002). *Glycyrrhiza glabra* contains a variety of substances.

In the traditional medicine system, the liquorice have been in clinical use for centuries. The roots of *Glycyrrhiza glabra* have antiulcer, expectorant, diuretic, laxative, sedative (Hikino, 1985), antipyretic (Lata et al., 1999), antimicrobial and anxiolytic activities (Ambawade et al., 2001). The main constituent of *Glycyrrhiza glabra* is glycyrrhizin which has antiviral (Ceremel atherosclerosis li et al., 1996) anti-inflammatory (Yokota et al., 1998) and antioxidant properties (Ju et al., 1989).

The root of *Glycyrrhiza glabra* contains various sugars (upto 18%), flavonoids, saponoids, sterols, amino acids, starch and gums. The effective ingredient in liquorice is mainly glycyrrhizin, a triterpenoid glycoside, which constitutes up to 14% of total soluble solids content (Baran & Fenercioglu, 1991) giving the characteristic sweet taste from the liquorice root. Glycyrrhizin has few calories and can be used in the form of ammonium glycyrrhizin (AG) or monoammonium glycyrrhizin (MAG) in the foods and beverages. AG is about 50 times as sweet as cane sugar, imparts a tan coloration and slight liquorice flavour. However, liquorice flavour and colour are repressed in MAG. The liquorice root extract has been widely

used in the nutriment industry as a sweetening agent (Cook, 1973). There is a growing commercial interest in using liquorice root extract in food foams. Foaming properties of liquorice extract influence the sensory quality and shelf-life of the final product (Dickinson, 1989). So, the liquorice has found widespread usage as a foaming agent in alcoholic and non-alcoholic beverages (Arestov, 1976), in confectionery products (Mansvelt, 1979), in halva and sweets (Kafka et al., 1970; Portnova et al., 1991). Commercially, the liquorice root extract is supplied in concentrated or powdered form for ease of transportation (Baran & Fenercioglu, 1991).

The medicinal and pharmacological uses of *Glycyrrhiza glabra* have been described in several studies (Arase et al., 1997; Davis & Morris, 1991; Takahara & Watanabe, 1994). There are a lot of efficacious compound in liquorice roots like Licochalcone known as a novel estrogenic flavonoid isolated from herb licorice root that was reported to show significant antitumor activity in various malignant human cell lines (Fu Y. et al 2004). The *Glycyrrhiza glabra* roots aqueous extract inhibits the in vivo and in vitro proliferation of Ehrlich ascites tumor cells. The angioinhibitory activity of *G. glabra* was confirmed by its inhibition of angiogenesis in in vivo assays, peritoneal and chorioallantoic membrane assay. The extract from the roots of *G. glabra* was reported that it may be a potential supplemental source for cancer therapy (Sheela, 2006).

Antimicrobial potential of *Glycyrrhiza glabra* roots were exhibited as antimicrobial activity against both Gram-positive and Gram-negative bacteria. Potential using of licorice as antitubercular agent were detected through systemic experiments and sophisticated anti-TB assay (Gupta et al., 2008).

Due to probable anti-inflammatory and antioxidant properties of liquorice about memory enhancement effect, *Glycyrrhiza glabra* show that it promise as a memory enhancing agent in all the laboratory models employed (Dhingra et al. 2004).

In study reviewed by Vibha et al., The liquorice helps in restoring liver function in patients suffering from hepatitis C. It is currently being assessed as a HIV infection treatment, as it slows the repetition of virus in culture cell (Vibha et al., 2009). In study realized about atherosclerosis, it was find effectively preventable properties of the liquorice extracts (Asgary et al., 2007). The behavior of *Glycyrrhiza glabra* extract use about hepatoprotective and antioxidant agent properties was supported in fish (Yin et al., 2011). Nonetheless, the skin

whitening (Lee et al. 1996), skin depigmenting (Thorel 1996), skin lightening (Nohata et al., 2005; Haris et al. 2007), antiaging, anti-erythemic (Donald et al. 1998), emollient (Chatterjee et al. 2005), anti-acne (Nam et al., 2003; Brooke et al. 2004), a potential cancer chemopreventive agent (Chin et al. 2007), cardioprotective effects (Haleagrahara et al. 2011) and photoprotection effects (Akhtar et al. 2011) were mainly attributed to liquorice extract. This extract has attenuates the hepatotoxic effect of CCl4 (Rajesh and Latha, 2004). The other significant properties is also that *Glycyrrhiza glabra* showed important antidepressant-like activity probably by inhibiting brain monoamine oxidase activity and subsequent increase in the brain monoamines. Therefore it can be a potent candidate for the management of depression (Chowdhury et al., 2011).

c. Economic important of *Glycyrrhiza glabra*

Glycyrrhiza glabra plant has been recognized worldwide as an important medicinal herb. The value of the licorice trade in 2007 was estimated at 42 million US$ (Parker 2007).

Table 1. Export and import amouts of *Glycyrrhiza glabra* in Turkey (Yoğunlu A. 2011).

Licorice		***Export amount***	***Import amount***
2007	**Amount (Ton)**	249	8
	Value (1000$)	266	121
2008	**Amount (Ton)**	67	5
	Value (1000$)	125	88
2009	**Amount (Ton)**	311	51
	Value (1000$)	471	104
2010	**Amount (Ton)**	292356	6
	Value (1000$)	523258	46

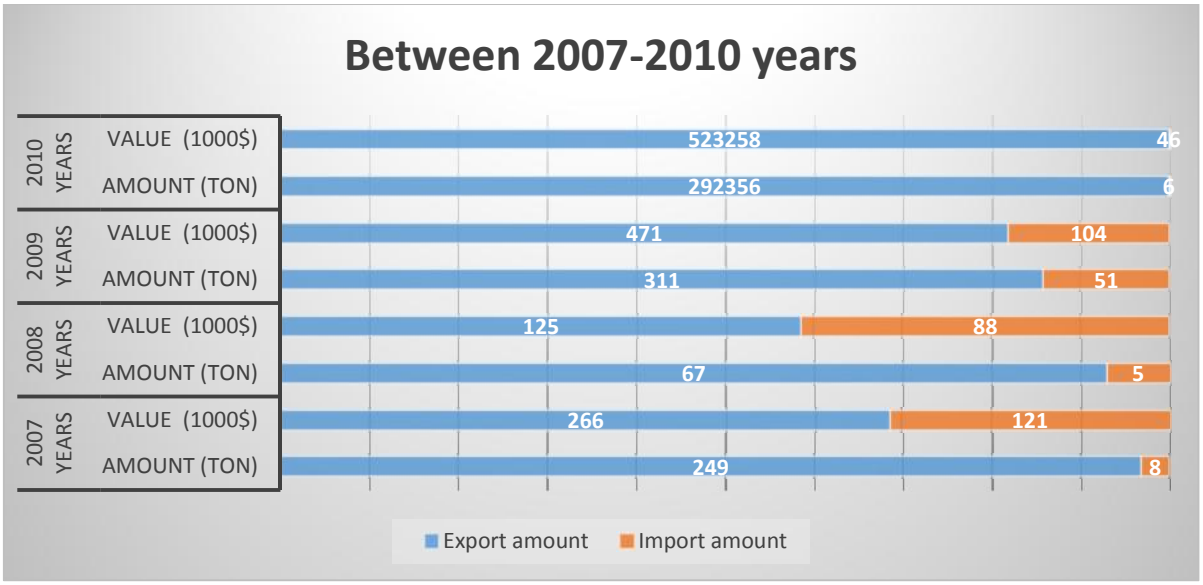

Figure 3. The Graphs of Export and import amounts of *Glycyrrhiza glabra* in Turkey

In Table 1, Export and import amounts of *Glycyrrhiza glabra* is displayed for Turkey. Because of above mentioned about the licorice, it is very importance for licorice in marketing share as a pharmaceutical agent, cosmetic, sweetener, food additive, flavor additive for tobacco, and confectionery food. However, this extract can be easily extracted from those residues.

As seen in the graphs of export and import amounts of *Glycyrrhiza glabra* in Turkey (Figure 3), this licorice is very important in marketing. So, the objective of this study were to enrichment the biological effective fractions by adsorptive bubble separation technique and no study has been done about enrichment of chemical composition from *Glycyrrhiza glabra* roots with bubble separation techniques so far.

II. PURPOSE OF THE STSM ABOUT ENRICHMENT OF THE GLYCYRRHIZIC ACID FROM LICORICE ROOTS (*Glycyrrhiza Glabra* L.) BY ISOELECTRIC FOCUSED ADSORPTIVE BUBBLE CHROMATOGRAPHY

The purposes of this STSM were:

- To conduct the enrichment of Glycyrrhizic acid ammonium salt known as biological effective fractions from licorice roots (*Glycyrrhiza glabra* L.) by isoelectric focused adsorptive bubble separation technique with different foaming agents.

- To learn isoelectric focused adsorptive bubble chromatography known as different isolation technique to obtained biological effective fractions after extraction of any non-wood forest products.
- To determine glycyrrhizic acid ammonium salt enriched from licorice roots (*Glycyrrhiza glabra* L.) by isoelectric focused adsorptive bubble separation technique with different foaming agents.To use and to set-up Isoelectric focused adsorptive bubble chromatography mechanism.
- To obtain experience about this bubble separation technique.
- To obtain information about the parameters affecting of adsorptive bubble separation technique like concentration, pH, shape of column, column length, column diameter, glass frit porosity and surface active agents.
- The explore and evaluate the installation cost in industry of adsorptive bubble separation technique in comparison to other isolation method.
- To evaluate the lowest cost in isolation technique of the licorice roots known as important in marketing share as a pharmaceutical agent, cosmetic, sweetener, food additive, flavor additive for tobacco, and confectionery food.
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- To publish the obtained results in an international journal.

It should be noted, that no study has been done so far on the enrichment of biological composition from *Glycyrrhiza glabra* roots with bubble separation techniques so far.

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III. DESCRIPTION OF THE WORK CARRIED OUT DURING THE STSM

Seperation biologically active natural products from medicinal herbs or useful plants is of great interest to the pharmaceutical and food industries. The separation technique such as solvent extraction or supercritical fluid extraction are usually employed for their isolation. Because of the ecosystem harm of organic solvents used, an alternative method of general interest is the so-called “foam fractionation”, a method based on adsorptive bubble separation. For the enrichment of surface-active substances, gases (e.g., nitrogen, oxygen, air, carbon dioxide, etc.) are introduced. So, enrich in the formed foam (Maas 1974; Short et al. 2005). The separation technique for biological effective compounds of foam fractionation by adsorptive bubble chromatograph is more effective, especially at low initial concentrations of substances (Short et al. 2005).

a. Solution and Reagents

The chemical solvent were used to HPLC spectroquality grade and other solvents of analytical grade. All of chemical solvents obtained from Sigma Chemicals Co. The glycyrrhizic acid ammonium salt standard was purchased from Sigma Chemicals Co. Foaming agents used as β-lactoglobulin, Albumin Bovine and Starch (soluble) were supplied from Sigma Chemicals Co. The water for the experiments was purified using a Milli-Q System (Millipore Corp.). *Glycyrrhiza glabra* roots were purchased from a local market.

b. Preparation and Extraction of Liquorice

The liquorice powder (10 g) was mixed with 300 mL distilled water. Mixtures were heated to 60°C under stirring for 4 hour and after cooling down, the solution was filtered with a fluted filter and the liquorice extract stored in the refrigerator at 4^{o}C until the absorptive bubble separation.

c. Adsorptive Bubble Chromatograph with Different Additional Substance

The equipment was established with a glass column (ID 18.5 mm, length 15 cm) with a porous frit (P 3, porosity 16-40 µm), flask (250 ml), initial solution column (ID 18.5 mm, length 15 cm) and volumetric flask (for flow measurement ml/min) (Figure 1). The attention was taken that the column highly clean and that the ground glass was free of fat. Gas bubbles were created by passing a stream of nitrogen through a glass frit dipping into the liquid pool at the bottom of the column. The bubble rises up the column and the liquid part of the bubble drains due to gravitation back to the starter solution. Because of foaming supporting, it was altered some foaming agent. The foam was collected in collection vessel at the upper part of apparatus. After bubble separation, enrichment samples collected in collection vessel were weighed. The separation takes place by means of an optional adsorption at the liquid gas interface of raised bubbles.

In the experiments, four bubble separation parameters were used with β-lactoglobulin, Albumin Bovine, Starch (soluble) preferred as foaming agents and without additive. For the every experiment, 70 ml of licorice root extraction amount was prepared, containing 30 mg foaming agents in 120 min foaming time. The carrier gas was nitrogen with a flow rate of 30 ml/min. The pH value was also 2,5. The enrichment ratios (E) were calculated as follows;

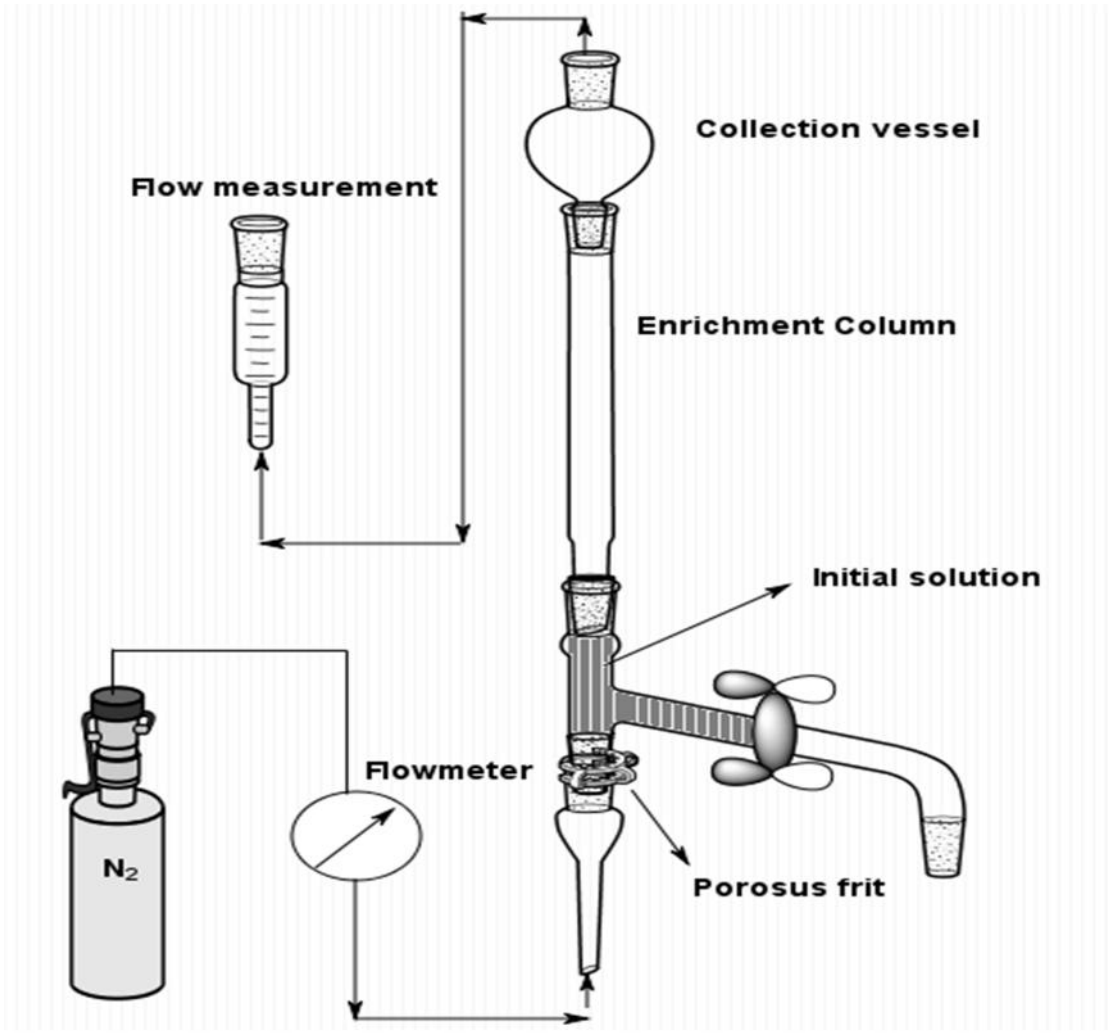

Figure 4. Scheme of the Adsoptive Bubble Seperation Aparatus

$$E = \frac{Cf}{CS}$$

Where, E is enrichment ratio, C_{foam} is concentration (mg/ml) of foaming after enrichment with bubble separation and C_{start} is initial concentration as mg/ml. The eluting fractions were collected at fixed intervals and after foam destruction with 1ml eluent (A), which used in HPLC as mobile phase subjected directly to analysis.

All the calibration curves were plotted based on linear regression analysis of the integrated peak areas (x) versus concentrations (y; mg/L, ppm) of the reference solution at three different

concentrations. Regression equation, retention time and correlation coefficient and calibration curve of glycyrrhizic acid ammonium salt in HPLC were shown in Table 2 and Figure 5

Table 2. Regression equation, retention time, correlation coefficient of reference compounds on HPLC

No	Sample Concantration (mg/L) (ppm)	Retention Time (min)	Regression equation[a]	Correlation coefficient (r^2)
1.	138			
2.	207	3,04	y = 0,0016x - 21,842	0,9917
3.	276			

[a] x: peak area of components, y: concentration of components

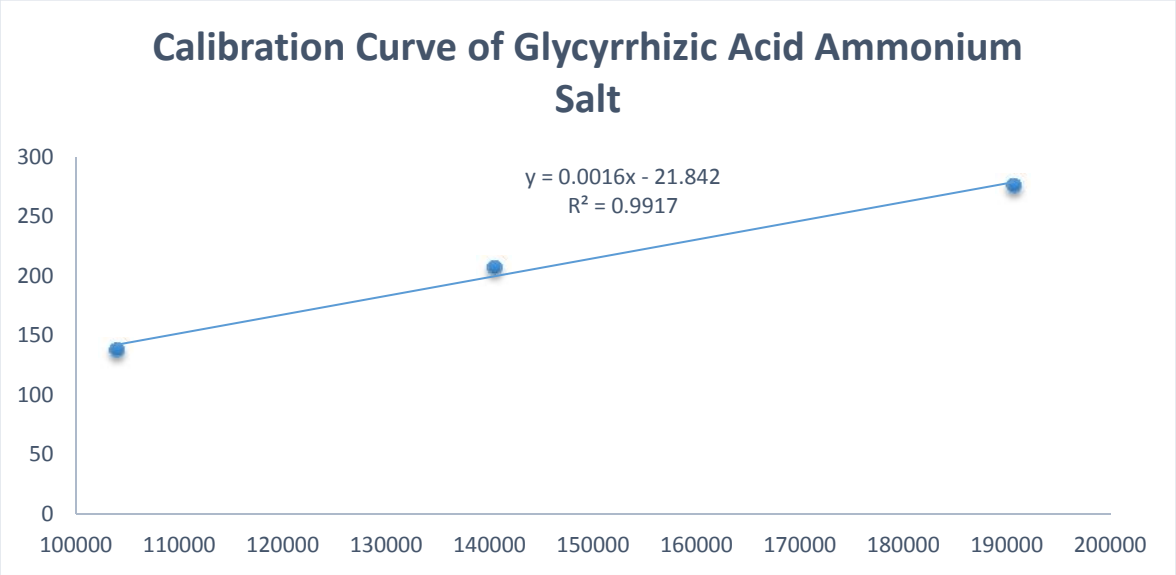

Figure 5. Calibration Curve of Reference Compounds on HPLC

The starting, remaining solution and extract foamed samples were analyzed by HPLC for identification and quantification of glycyrrhizic acid ammonium salt.

d. HPLC Instrumental

A Gynkotek 480 equipped with a Rheodyne 8125 injector and 20 µL sample loop, a Gynkotek UV-detector (UVD 340 s, wavelength was selected as 254 nm), a Kromasil 100 C 18 column (Knauer, Germany: 5 µm, 25064.6 mm; column temperature 258 ^{0}C) and a uniflow degasser DG-1310 were used. The eluent (A) contained 210 mL of methanol, 210 mL of acetonitrile, 174 mL of distillated water and 6 ml glacial acetic acid without gradient elution, at a flow rate of 1 mL min^{-1}.

IV. DESCRIPTION OF THE MAIN RESULTS

The licorice root extracts gave ample foam during bubble separation, when β-lactoglobulin, Albumin Bovine, Starch (soluble) (30 mg for everyone) were used as foaming in 70 ml the liquorice extract. The foam without additional of a surface active substance were weak in the liquorice extract. This situation was probably due primarily to the reduction of surface tension.

Table 3. Enrichment ratios and start, foam, residual extract concantrations (mg/ml) of *Glycyrrhiza glabra* with β-lactoglobulin, Albumin Bovine, Starch (soluble) preferred as foaming agents and without additive.

Amount of Licorice Root Extraction	Additional Substance (mg)	Foam Volume (ml)	Start (C) (mg/ml)	Foam (C) (mg/ml)	Residual (C) (mg/ml)	Enrichment Ratio (E)
Exp 1	30mg β-Lactoglobulin	4,19ml and 12ml Eluent A	0,012	4,42	0,014	368,3
Exp 2	29,9mg Albumin Bovine	8,14ml and 8ml Eluent A	0,024	2,17	0,031	90,4
Exp 3	30 mg Starch (soluble)	5,33ml and 10ml Eluent A	0,348	3,26	0,026	9,4
Exp 4	Without additive	10,64ml and 9ml Eluent A	0,36	2,10	0,026	5,9

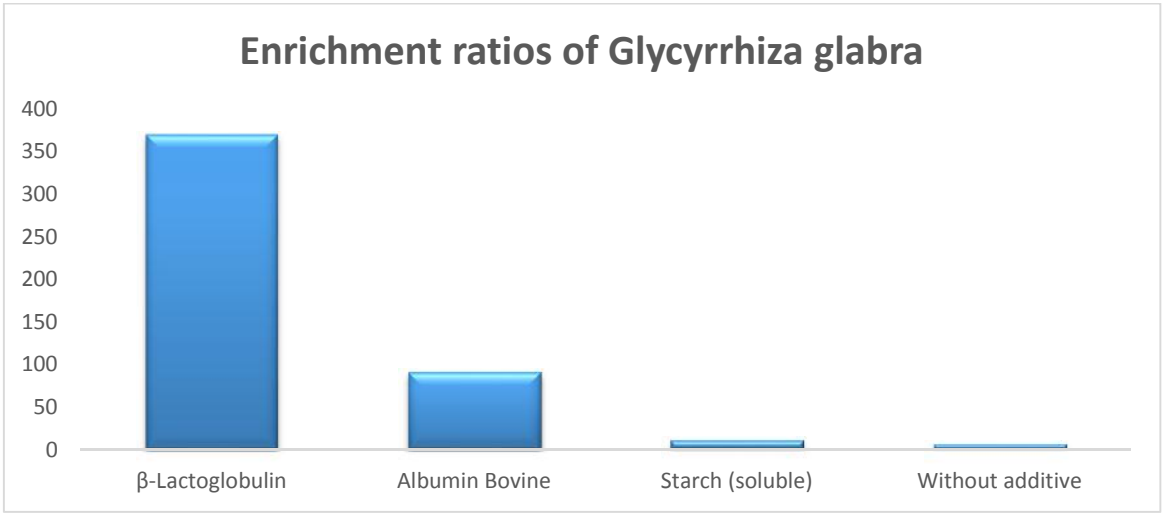

Figure 6. Enrichment ratios of *Glycyrrhiza glabra* with β-lactoglobulin, Albumin Bovine, Starch (soluble) preferred as foaming agents and without additive.

The licorice root extraction amount, pH value, foaming agent amount, foaming time and gas flow rate were taken as optimum on the enrichment. The foaming agent type was used as changeable, while others was stable. During all experiments, a higher or lower enrichment rates of glycyrrhizic acid ammonium salt into the foam fraction could be observed **(Table 3, Figure 6)**. Varying the additional substances influenced the enrichment ratios and yields.

In conclusion, these results indicated that it is possible to enrichment of glycyrrhizic acid ammonium salt from *Glycyrrhiza glabra* root extract almost quantitatively by isoelectric focused adsorptive bubble separation technique at the both additive and without adding a surfactant. The enrichment glycyrrhizic acid ammonium salt obtained from *Glycyrrhiza glabra* root extract may use in industry as pharmaceutical agent, cosmetic, sweetener, food additive and confectionery food.

V. FUTURE COLLABORATİON WITH HOST INSTITUTION

Host institution (O. Prof. Dr. Dr. Harun PARLAR) has a lot of separation techniques knowledge about isolation of biological effective fractions from any non-wood forest products and their chemical characterization by any instrument machine like HPLC-UV, HPLC-DAD, HPLC-ELCD, HPLC-MS/MS and GC-MS. I will contact about isoelectric focused adsorptive bubble chromatography technique due to my next studying. Because, this separation technique needs experiences.

About future collaboration, my other aim is that Host institution has discovering new separation technique of biological effective fractions from any plant materials. In the same time, Host institution has patent about his new separation technique. Name of this new separation technique is tweezing adsorptive bubble separation (TABS) technique. With this method, it is only pick for biological effective fractions and was to apply alternatively the so-called tweezing adsorptive bubble separation (TABS) technique for the enrichment and isolation. For example, Parlar et al. find that in principle it is possible to effectively isolate insulin from respective solutions by using TABS technique and this technique is more eco-friendlier and cost-effective than other methods. (Nicolal et al. 2008). A newly developed tweezing-adsorptive bubble separation method for the enrichment of metalloenzymes has high effective (Gerken et al. 2005).

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