

A Convenient HPLC/ELSD Method for the Quantitative Analysis of Betaine in *Lycium chinense*[†]

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Abstract – In order to facilitate the quality control of betaine from the fruits of *Lycium chinense*, we have developed a rapid and simple method for quantitative determination. Determination was achieved on a Discovery C18 column with an isocratic solvent system of 0.32% perfluoropentanoic acid aqueous-acetonitrile at a flow-rate of 0.5 mL/min and detected an ELSD. The method was reproducible with intra- and inter-day variations of less than 6% (R.S.D). The recoveries were in the range of 90.01~100.05%. The method turned out to be fast and simple, furthermore, to have a good selectivity and sensitivity for the quantity determination of betaine in the fruits of *L. chinense*.

Keywords – *Lycium chinense*, Solanaceae, Betaine, HPLC-ELSD detector

Introduction

The fruits of *Lycium chinense* Miller (Solanaceae), widespread in Northeast Asia, have been used as a tonic in traditional Chinese medicine. It has been added into tea and used as herbal drug in Asia for more than 2000 years. Several volatile, steroidal and alkaloidal compounds in this plant are known to have various bioactivities such as improving stamina, tranquillizing, and thirst-quenching (Funayama *et al.*, 1980; Noguchi *et al.*, 1984; Sannai *et al.*, 1983; Yang *et al.*, 1987). Betaine is a natural amino-acid and one of the major constituents of *L. chinense*. It is reported that this fruit plays a role in reducing blood levels of homocysteine, a toxic by-product of the amino-acid metabolism which probably promotes atherosclerosis and osteoporosis (Gahl *et al.*, 1988; Selhub, 1999; Wendel *et al.*, 1984). In animal studies, betaine supplementation has shown protection against chemical damage to the liver (Barak *et al.*, 1993; Junnila, *et al.*, 1998; Murakami *et al.*, 1998).

Several other analytical approaches for the determination of betaine in fruits of *L. chinense* or in other biological sources have been published, such as through liquid chromatography and gas chromatography/mass

spectrometry (GC/MS) (Mar *et al.*, 1995). However, betaine does not have a suitable chromophore for UV detection or a suitable polarity for separation through an analytical column. The sensitivity and resolution degree of the published methods are not suitable enough to be used in a quantitative analysis of betaine (Kikuchi *et al.*, 1993; Shin *et al.*, 1999). Most of the existing methods do not have a simplified step for the sample preparation. Therefore, the betaine analysis method from *L. chinense* requires a simplified sample preparation step that has a better resolution degree and a lower-end detection or a quantitation limit.

We have developed a new convenient analysis method that can determine the quantity of betaine in *L. chinense* fruits using HPLC coupled with an evaporative light scattering detector (ELSD) and an ODS column with an ion-pairing reagent (perfluoropentanoic acid, PFPA) as mobile phase. The present paper describes the analysis method and the validation of this method.

Experimental

Apparatus – For this experiment, we used the Waters Alliance HPLC system with 2695 Separation Module, and the 2420 ELS Detector (Waters, MA, USA). Chromatographic separations were performed on an ODS column (4.6 mm ID × 250 mm, 5 μm, Discovery C18, SUPELCO, PA, USA). The Empower Pro Chromato-

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graphy Data Software (Waters, MA, USA) was used for the data acquisition and processing as well as for regulating the HPLC system. In order to confirm the analytical specificity, the Waters Acquity UPLC system coupled with the TQ Detector (Waters, MA, USA) was used. The column used for this specific purpose was an Acquity BEH C₁₈ (2.1 mm I.D. × 100 mm, 1.7 μm, Waters, MA, USA).

Materials and chemicals – The dried fruits of *L. chinense* were a contracted cultivation by Korea Ginseng Corporation (Daejon, Korea). The voucher specimens were deposited in the specimen custody freeze warehouse of KGC Central Research Institute (Daejon, Korea). The standard material of betaine (> 95%) was purchased from ChromaDex (CA, USA), perfluoropentanoic acid (97%) from Sigma-Aldrich (WI, USA) and acetonitrile (HPLC grade) was obtained from J. T. Baker (NJ, USA). For the treating water (with a resistivity more than 18 MΩ Cm) the Ultra Pure water system (Sinhan Science Tech, Daejon, Korea) was used throughout the experiment.

Preparation of sample and standard solutions – The coarsely powdered sample (0.5 g) was extracted with distilled water (80 mL) under a water bath for 3 h at 80 °C. After filtering the extract with a filter paper, it was poured into 100 mL volumetric flask. Then distilled water was filled until its maximum volume and afterwards, the suspension was filtered through a 0.22 μm MILLEX GV syringe filter (Millipore, MA, USA) prior to the injection (20 μL) into the HPLC/ELSD system. This stock solution of betaine was prepared in water (1 mg/mL) and standard solutions of 0.006, 0.012, 0.025, 0.05 and 0.1 mg/mL were produced by diluting the stock standard with water.

High performance liquid chromatography – The HPLC based separation of betaine for quantitative analysis was performed using a reverse phase system. Afterwards, an ODS column (4.6 mm ID × 250 mm, 5 μm, Discovery C18, SUPELCO) with acetonitrile (5%) in water (0.32% perfluoropentanoic acid) at a flow rate 0.5 mL/min was used at 60 °C. The injection volume was 20 μL. The ELSD nebulizer temperature was at 20 °C, the level of the drift tube 70 °C, the pressure of gaseous N₂ 30 psi, and the data gain value 3.

Identification by MS/MS detector – The Waters ACQUITY UPLC with a TQ Detector system was used to confirm the method specificity. Separation was performed on the reverse-phase column (ACQUITY BEH C18, 2.1 mm I.D. × 100 mm, 1.7 μm, Waters) with an eluting mobile of 0.1 % formic acid in distilled water - acetonitrile (90 : 10). The flow rate was at 0.4 mL/min and the injection volume was 2 μL. The spectra were

acquired in the positive mode. Source and desolvation temperatures were at 120 °C and at 450 °C, respectively, and the gas flow of desolvation and cone 800 L/h and 50 L/h, respectively. Capillary voltage was set at 3 kV. Betaine was detected in multiple reaction monitoring (MRM) modes using mass-to-charge (m/z) transitions of precursor and product ions. The parent ion of betaine was observed at m/z 118 and the product ion at m/z 59 with a cone voltage 18 kV.

Method Validation – The identification of betaine was carried out by comparing the retention times of the peak in the standard with the corresponding ones in the sample solutions measured through the HPLC-ELSD method, and the quantitative values of betaine measured through the HPLC-ELSD method and the UPLC-MS/MS method. The calibration curve was generated by plotting the peak areas vs. the concentrations of standard solutions. Each standard was analyzed three times. This repeatability was chosen in order to determine the accuracy of the method. For this, three concentrations (0.25, 0.5, 1 g/100 mL) and three replicates of each concentration were sampled for the repeatability test. The limits of detection (LOD) and quantification (LOQ) for betaine was determined at the standard deviation of the y-intercepts of the regression lines to the slope of the calibration curves ratio (s/S) of 3.3 and 10, respectively. Variations were expressed by the relative standard deviations (R.S.D.s). The recovery test for improving the accuracy of this method will be described with the next procedure. After adding the standards which correspond to 60, 100 and 140% of the amounts of betaine in the *L. chinense* fruit powder, the sample solutions were prepared according to the method in section - Preparation of sample and standard solutions. The quantity of each analyte was subsequently obtained from the corresponding calibration curve.

Results and discussion

Various columns and solvents were investigated to enhance the resolution and sensitivity for betaine detection in *L. chinense*. However, due to the ionic properties and the weak hydrophobic character of betaine amino silica (NH₂-silica), the reverse phase and the porous graphitic carbon column were not efficient enough to separate betaine from other constituents in *L. chinense* by elution with deionized water and organic solvents. However, the presented method (section Experimental) using an ion-pair reversed-phased liquid chromatography (IP-RPLC) with long chain perfluoropentanoic acid (PFPA) as the volatile ion-pairing reagent on octadecyl

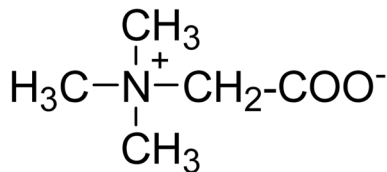


Fig. 1. Chemical structure of betaine.

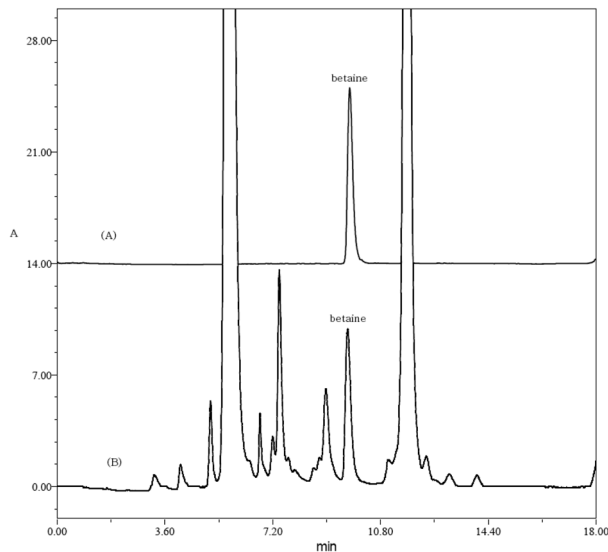


Fig. 2. Comparison of two chromatographies, the betaine standard (A) and the extract of *L. chinense* (B). Mobile phase; acetonitrile (5%) in water (0.32% perfluoropentanoic acid). flow rate 0.5 mL/min, column temperature; 60 °C, ELSD nebulize temperature; 70 °C, level of the drift tube; 20%, pressure of gaseous N₂; 30 psi.

silica (ODS) column and an evaporative light scattering detector (ELSD), includes a simple preparation of the sample solution, had a good resolution degree and a low detection limit. In order to determine the specificity of betaine peaks, the quantitative value of the sample was compared with the MS/MS and ELSD. As a result, the quantitative value of betaine in the sample was the same as in the standard materials (Fig. 2). In particular, the quantitative values by the ELSD and the MS/MS detector were almost identical with an inaccuracy of 3.79 %. Thus, the analysis of the specificity of betaine was confirmed.

The linearity calibration curves were obtained over the concentration range of 0.006–0.1 mg/mL with three replicates of each concentration. The correlation coefficient of the calibration function ($y = 43496x - 486031$, $r > 0.997$) indicated a good correlation between the standard concentrations and their peak areas within the test range. The LOD and LOQ were 18 and 54 ng, respectively. The preciseness was evaluated by the means of repeatability measures. For this, three concentrations (0.25, 0.5, 1.0 g)

and three replicates of the analysis on the same powdered fruits of *L. chinense* sample were used, and expressed in terms of relative standard deviation (R.S.D). The variations of the measured values were less than 6 % (R.S.D) and therefore, this method was proved to be precise. The accuracy of the developed method is defined by the closeness of the test results to the true value. From the results, the R.S.Ds of the overall recoveries were < 5% and the recovery rates were at 90.01–100.05% which showed that the method was high accuracy. Therefore, this method showed great reliability on obtaining accurate results. All the results above indicated that this analysis method was rapid, precise, accurate, and sensitive for quantitative determination of betaine from fruits of *L. chinense*.

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