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Original Article

A feasible and practical ^{1}H NMR analytical method for the quality control and quantification of bioactive principles in Lycii Fructus

Li-Ying Hsieh [a,1], Hsiu-Hui Chan [a,1], Ping-Chung Kuo [b,1], Hsin-Yi Hung [b,**], Yue-Chiun Li [c], Chao-Lin Kuo [d], Yong Peng [e], Zhong-Zhen Zhao [f], Daih-Huang Kuo [g], I. -Wen Sun [a], Tian-Shung Wu [b,g,*]

[a] Department of Chemistry, National Cheng Kung University, Tainan 701, Taiwan
[b] School of Pharmacy, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
[c] Graduate Institute of Biotechnology, National Chung-Hsing University, Taichung 402, Taiwan
[d] Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung 401, Taiwan
[e] Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100094, China
[f] School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China
[g] Department of Pharmacy, College of Pharmacy and Health Care, Tajen University, Pingtung 907, Taiwan

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ABSTRACT

Lycii Fructus, a solanaceous drug, is widely used as functional foods and in Traditional Chinese Medicine. Samples collected from different regions of China have been found to be not identical in chemical compositions which might affect the biological activities. Although many chromatographic and spectrometric methods have been reported to determine the concentration of betaine and other bioactive amino acids, disturbance resulted from other polar substances with low UV-absorbance and expensive mass facilities reduced the applicability of these techniques. In the present study, the strong cation exchange solid phase extraction procedure incorporated with ^{1}H NMR was successfully developed as a rapid and reliable method that can simultaneously determine betaine, citric acid, threonine, alanine, and proline in various Lycii Fructus. In addition, ERETIC 2 method based on PULCON principle was also applied and compared with conventional method. This feasible and practical method offers a very powerful tool for the quality control of commercial Lycii Fructus from different sources.

Abbreviations used: ERETIC 2, Electronic Reference To access In vivo Concentration 2; SCX-SPE, strong cation exchange solid phase extraction; PULCON, PUlse Length-based CONcentration determination.

* *Corresponding author.* School of Pharmacy, National Cheng Kung University, No. 1, University Road, Tainan 701, Taiwan. Fax: +886 6 2740552.
** *Corresponding author.*
E-mail address: tswu@mail.ncku.edu.tw (T.-S. Wu).
[1] These authors contributed equally.

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1. Introduction

Wolfberry fruits, a solanaceous origin, also known as Lycii Fructus are rich in polysaccharides, alkaloids, carotenoids, fatty acids, essential trace elements, and amino acids and have been used for centuries in Traditional Chinese Medicine to nourish the liver and kidney and protect the eyesight. Lycii Fructus had been demonstrated to display various biological functions, such as anti-inflammatory and hepatoprotective activities, and the prevention of tumor growth [1–3]. According to Taiwan Herbal Pharmacopeia, *Lycium barbarum* L. and *L. chinense* Mill. from Ningxia province of China are the authentic medicinal herbs of Lycii Fructus [4]. Different sources of Lycii Fructus had been reported to show different chemical compositions which might affect the biological activities. Therefore, establishment of composition fingerprint profile of Lycii Fructus for quality control is warranted. Currently, the quaternary ammonium cation betaine has been used as one of the biomarkers for Lycii Fructus identification and quality control of commercial products [1]. Betaine is one of the major components in the fruits of *Lycium* species and exhibits anti-inflammatory, hepatoprotective, and anti-tumor activities [5–7]. Although high performance liquid chromatography (HPLC) [8,9] had been reported to determine the components of Lycii Fructus, betaine was difficult to be distinguished from other amino acids or citric acid due to lack of UV-chromophore. In addition, liquid chromatography–mass spectrometry (LC-MS) method [10–12] which can solve the detection limit of low UV-absorbance substances still requires expensive mass facilities and tedious pretreatment procedures. High-resolution nuclear magnetic resonance (NMR) spectroscopy has become an increasingly important quantitative tool, providing high specificity and sensitivity for detecting natural products, even those without UV chromophore [13–17]. In our previous studies, ^{1}H NMR spectroscopy was successfully used to quantify bioactive constituents from many natural products, including *Coptidis Rhizoma*, *Codonopsis Radix*, *Ginkgo Folium*, *Phellodendri Cortex*, and *Nothapodytes foetida* [18–22]. Herein we report a rapid quantitative ^{1}H NMR method that can simultaneously quantify betaine, citric acid, and other amino acids with low UV-absorbance such as threonine, alanine, and proline in Lycii Fructus, respectively. To quantify these charged molecules with low UV-absorbance in Lycii Fructus, the strong cation exchange solid phase extraction (SCX-SPE) procedure was utilized to trap the desired molecules in the aqueous extracts of *Lycium* fruits from various sources and then the ^{1}H NMR method was performed. With the aid of the developed method, the quantity of bioactive constituents in the commercial products of Lycii Fructus could be analyzed quickly and conveniently.

2. Materials and methods

2.1. Chemicals and materials

Deuterium oxide (D_2O, 99.98%), maleic acid, and succinic acid were obtained from Sigma–Aldrich (Milwaukee, WI, US). The reference compounds (betaine, citric acid, proline, threonine, and alanine) were purchased from Merck (Darmstadt, Germany). The ultrapure water (H_2O) was prepared with Milli-Q water purification system (Millipore, Bedford, MA, US). The analytical cartridge column was using Thermo Fisher Scientific (Waltham, MA, US) HyperSep SCX strong cation exchanger SPE columns (2000 mg). *Lycium* fruit samples 1–23 and 27–42 were purchased from the markets in China. Samples 24–26 were collected in Shanxi province of China in Oct, 2009. All samples were purchased and collected by Dr. Yong Peng. The materials were identified by Prof. C. S. Kuoh (Department of Life Science, National Cheng Kung University), and voucher specimen (TSWu 20100708-01-42) have been deposited in the Department of Chemistry, National Cheng Kung University, Tainan, Taiwan.

2.2. Sample preparation

Lycium fruit samples were air-dried at room temperature for three days and pulverized. Five grams of samples was extracted three times with 50 mL H_2O by sonication for 30 min. The afforded solution was combined and filtered through a 0.45 μm membrane filter. The aqueous filtrate was transferred to a 250 mL volumetric flask and diluted to 250 mL with H_2O. The strong cation exchanger SPE column was activated with 8 mL 0.1 N acetic acid and washed with 200 mL Milli-Q water to obtain pH 7. Then, 25 mL of diluted solution was passed through the SPE cartridge at a flow rate of 4 mL/min, and the cartridge was then washed with 30 mL H_2O. The internal standard 0.5 mg maleic acid was added to the elution solvent, and the solvent was evaporated to dryness *in vacuo* to afford LYW. LYW was dissolved in 0.6 mL of D_2O for NMR analysis. The SPE cartridge was then washed with 25 mL 5% ammonia water. The internal standard 0.5 mg succinic acid was added to the elution buffer and evaporated to dryness *in vacuo* to obtain LYN. LYN was also dissolved in 0.6 mL of D_2O for NMR analysis.

2.3. ^{1}H NMR spectrometric parameters

^{1}H NMR spectra were recorded on a Bruker AVANCE III 400 MHz spectrometer in D_2O solvent systems, and all chemical shifts are reported in parts per million (ppm, δ). For each sample, 100 scans were recorded with the following parameters: spectrum resolution 0.39 Hz/point; spectral width, 6393.862 Hz; A 90° pulse was used to obtain the maximum sensitivity; relaxation delay, 20 s; and acquisition time, 2.56 s. For quantitation the peak area was used, and the start and end points for the integration of each peak were selected manually. In addition, quantitative determination (qNMR) of targeted molecules in reference materials has been established using the ERETIC 2 methodology (electronic reference to access in vivo concentrations 2) based on the PULCON principle (pulse length based concentration determination). The NMR parameters for ERETIC 2 are the same as mentioned above. Bruker TopSpin version 3.0 software was used.

The amounts of citric acid were calculated by the following formula:

$$\{(0.5\ \text{mg}/116) \times [(\mathbf{A1} \times 2)/\mathbf{N}] \times \mathbf{MW}$$

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