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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:

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

The amounts of betaine, threonine, alanine, and proline were calculated by the following formula:

$$\{(0.5\ \text{mg}/192) \times [(\mathbf{A2} \times 4)/\mathbf{N}] \times \mathbf{MW}$$

A1: the area ratio of target signal to internal standard maleic acid;
A2: the area ratio of target signal to internal standard succinic acid;
N: the number of proton atom in target signal;
MW: molecular weight (maleic acid (IS): MW = 116; citric acid: MW = 192; succinic acid (IS): MW = 118; threonine: MW = 119; alanine: MW = 89; betaine: MW = 117; proline: MW = 115)

2.4. *Recovery test and limit of detection*

Known amounts of pure betaine and citric acid were spiked to samples to evaluate the % recovery. The recovery samples and a blank recovery sample were processed and analyzed as described above.

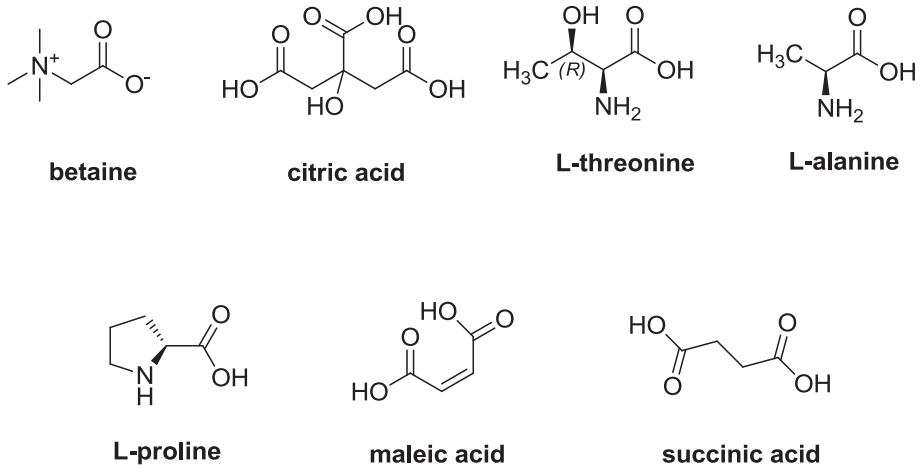

Fig. 1 – The structures of analytical compounds and internal standards.

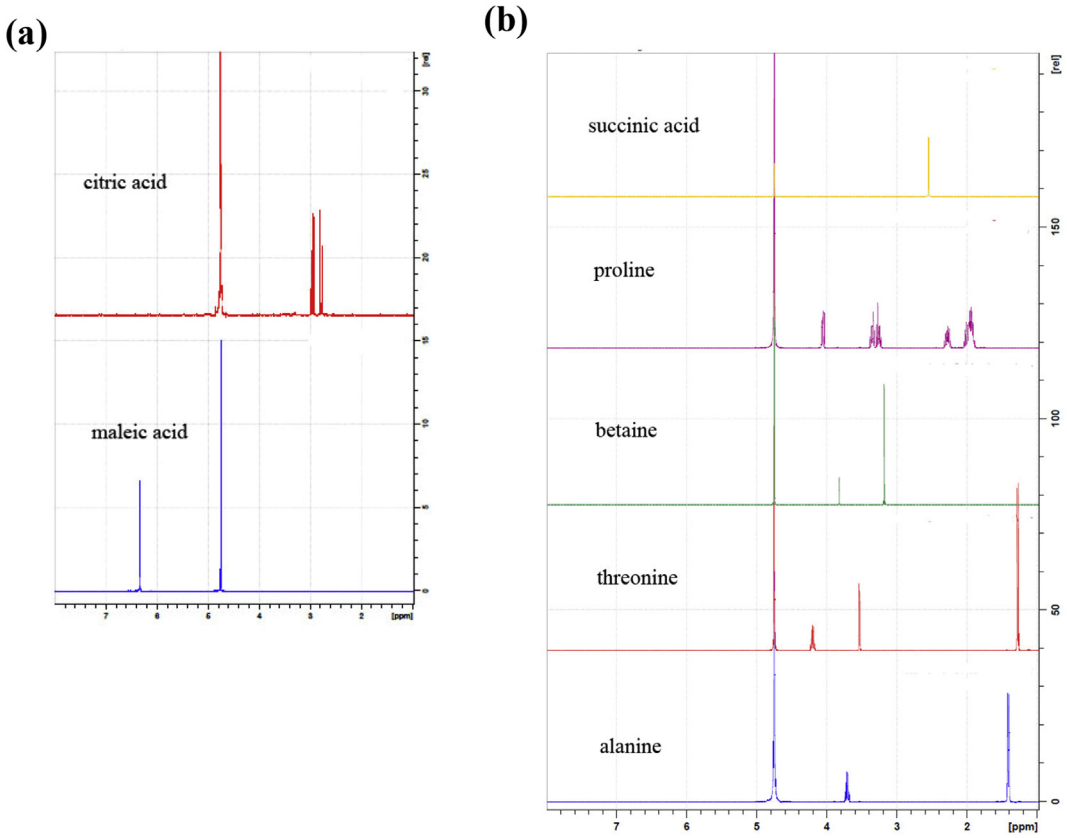

Fig. 2 – ^{1}H NMR spectra of (a) internal standard (maleic acid) and citric acid (b) internal standard (succinic acid), proline, betaine, threonine, and alanine. All compounds were detected in D_2O system.

3. Results and discussion

The structures of the target compounds including betaine, citric acid, proline, threonine, alanine, and internal standards including maleic acid, and succinic acid, are shown in Fig. 1. These target compounds were effectively extracted with water by sonication and then collected through SCX-SPE. Moreover, D_2O (residue solvent peak δ_H 4.75 ppm) was used as NMR solvents to effectively dissolve all the target compounds. The analysis of their respective ^1H NMR spectra revealed that the signals of betaine (δ 3.21, 9H), citric acid (δ 2.81, 2.99, 2H), proline (δ 4.08, 1H), threonine (δ 1.28, 3H), and alanine (δ 1.43, 3H) were well separated from the other signals, and thus these signals were selected as the target peaks for quantitation (Fig. 2). In addition, maleic acid (δ 6.36, 2H) and succinic acid (δ 2.37, 4H) in D_2O solvent system were chosen as the internal standards in the samples except those detected by ERETIC 2 and the signals of internal standards were also well separated from the target signals.

The calibration curve for each compound using the ratio of the peak area of the compound and the internal standards, were determined in the range of 0.0625–2.0 mg/mL to evaluate the accuracy of this method at different concentrations. Betaine, citric acid, proline, threonine, and alanine were found to show good linearity with r^2 higher than 0.99 (0.9961, 0.9979, 0.9902, 0.9955 and 0.9956, respectively). In comparison, the previously reported chromatographic method [7,8] only showed linearity at the concentrations of 400–2000 ppm.

Table 1 – Contents of betaine, citric acid, threonine, alanine, and proline in 42 samples from the fruits of *Lycium* species[a].

NO.	Collection place	Betaine	Citric acid	Threonine	Alanine	Proline
1	Ningxia, China	0.44 (1.56)	1.17 (0.41)[c]	0.03 (6.67)	0.15 (0.34)	0.61 (4.14)
2	Ningxia, China	0.65 (0.11)	1.27 (0.19)	0.03 (3.94)	0.19 (1.01)	0.71 (1.12)
3	Ningxia, China	0.59 (1.78)	2.15 (6.43)	0.01 (3.84)	0.03 (6.14)	0.41 (2.08)
4	Ningxia, China	0.21 (4.15)	1.42 (0.79)	0.06 (6.59)	0.06 (6.60)	0.12 (2.43)
5	Ningxia, China	0.84 (2.44)	2.51 (2.00)	–	0.20 (1.37)	0.39 (7.36)
6	Ningxia, China	0.70 (1.01)	1.59 (1.01)	0.02 (1.01)	0.25 (1.01)	0.44 (1.01)
7	Nei Mongol, China	0.92 (7.35)	1.83 (1.44)	0.01 (6.40)	0.16 (3.90)	0.40 (6.14)
8	Nei Mongol, China	0.67 (2.52)	1.31 (0.80)	0.02 (7.33)	0.25 (3.55)	0.32 (6.89)
9	Nei Mongol, China	0.81 (1.85)	1.07 (0.77)	0.03 (4.51)	0.15 (2.05)	0.65 (1.22)
10	Nei Mongol, China	0.48 (0.51)	1.07 (0.10)	0.02 (1.21)	0.10 (1.12)	0.46 (7.56)
11	Qinghai, China	0.50 (8.64)	1.14 (4.52)	0.03 (5.58)	0.15 (8.16)	0.71 (8.80)
12	Qinghai, China	0.61 (4.19)	0.89 (0.11)	0.01 (5.55)	0.11 (3.98)	0.46 (2.96)
13	Qinghai, China	0.59 (0.34)	1.41 (0.21)	0.03 (6.53)	0.22 (1.08)	0.85 (0.86)
14	Qinghai, China	0.52 (1.49)	0.89 (0.53)	0.01 (3.65)	0.13 (2.09)	0.85 (2.20)
15	Qinghai, China	0.94 (0.73)	1.41 (1.46)	0.04 (2.81)	0.15 (0.99)	0.92 (1.65)
16	Gansu, China	0.61 (8.48)	1.31 (3.07)	0.03 (0.87)	0.15 (3.43)	0.49 (8.47)
17	Gansu, China	0.70 (2.34)	1.08 (1.26)	0.02 (4.80)	0.14 (0.66)	0.18 (6.77)
18	Gansu, China	0.59 (3.42)	1.40 (0.41)	0.02 (3.01)	0.12 (4.29)	0.17 (7.90)
19	Gansu, China	1.04 (1.50)	0.79 (1.02)	0.04 (7.80)	0.20 (3.85)	0.67 (3.04)
20	Xinjiang, China	1.01 (3.94)	1.24 (1.68)	0.01 (8.30)	0.20 (3.61)	0.21 (2.43)
21	Xinjiang, China	0.74 (1.80)	1.33 (1.96)	0.01 (0.71)	0.14 (1.58)	0.28 (4.66)
22	Xinjiang, China	0.99 (3.47)	0.81 (5.16)	0.04 (4.94)	0.22 (5.96)	0.75 (7.22)
23	Shanxi, China	1.09 (5.95)	0.89 (4.63)	0.02 (7.98)	0.21 (2.75)	0.47 (5.46)
24[b]	Shanxi, China	0.77 (8.41)	1.95 (0.66)	0.02 (7.14)	0.06 (6.23)	0.05 (3.17)
25[b]	Shanxi, China	0.80 (0.58)	1.95 (0.45)	0.04 (3.72)	0.12 (3.91)	0.03 (4.26)
26[b]	Shanxi, China	0.46 (1.42)	1.25 (0.37)	0.04 (6.42)	0.08 (1.43)	–
27	Shaanxi, China	0.62 (2.42)	1.16 (0.57)	0.01 (5.94)	0.13 (7.06)	0.21 (8.06)
28	Sichuan, China	0.38 (2.80)	1.59 (1.47)	0.04 (8.03)	0.08 (1.19)	0.06 (7.25)
29	Hebei, China	0.51 (6.26)	0.94 (4.98)	0.02 (6.79)	0.06 (2.53)	0.06 (7.36)
30	Beijing, China	0.77 (2.03)	1.50 (1.15)	0.01 (3.74)	0.12 (3.59)	0.21 (5.13)
31	Beijing, China	0.88 (1.93)	1.60 (1.34)	0.01 (7.59)	0.16 (2.48)	0.27 (8.76)
32	Beijing, China	0.62 (2.47)	1.32 (3.62)	0.01 (7.86)	0.09 (3.27)	0.04 (8.66)
33	Beijing, China	0.95 (3.13)	2.10 (1.48)	0.01 (4.29)	0.17 (2.53)	0.17 (7.55)
34	Beijing, China	0.77 (6.29)	1.83 (0.43)	0.02 (7.17)	0.14 (2.19)	0.15 (8.67)
35	Beijing, China	1.17 (3.91)	1.77 (0.58)	0.02 (5.83)	0.25 (2.33)	0.28 (7.26)
36	Beijing, China	0.54 (1.21)	1.25 (1.61)	0.01 (6.29)	0.14 (1.16)	0.29 (7.98)
37	Beijing, China	0.78 (1.55)	0.98 (1.57)	0.02 (4.08)	0.19 (3.16)	0.11 (3.42)
38	Beijing, China	0.81 (2.60)	1.25 (1.11)	0.03 (4.63)	0.24 (5.73)	0.58 (5.03)
39	Beijing, China	0.83 (8.06)	1.28 (1.45)	0.05 (6.78)	0.27 (6.17)	1.51 (5.80)
40	Beijing, China	0.64 (5.84)	1.32 (2.25)	0.01 (2.46)	0.15 (7.74)	0.48 (7.99)
41	Beijing, China	0.57 (5.74)	1.26 (4.37)	0.01 (5.55)	0.13 (7.27)	0.32 (1.12)
42	Beijing, China	0.77 (2.67)	1.06 (1.85)	–	0.14 (1.20)	0.09 (6.70)

[a] Recorded on % (w/w) of *Lycium* species.
[b] Samples 24–26 were *L. chinense* samples, and others were *L. barbarum*.
[c] % RSD, all experiments were based on triplicate measurements.

Relative recovery tests were conducted using three different quantities of the standards. The average relative recoveries were calculated as ratios by spiking known amounts of pure betaine and citric acid, and the experiments were performed in triplicate. The average relative recoveries of betaine and citric acid were determined as 99.34 ± 4.1% and 99.57 ± 3.5%, respectively. These data indicated that the reproducibility and recovery of the analysis process were acceptable. The precision and recovery examinations all displayed that the established ^{1}H NMR methods were valid for the quantitative determination of the target compounds.

In addition, the *Lycium* fruit samples were only extracted, passed through SPE cartridge and re-dissolved in D_2O for NMR analysis. The simple pre-treatment steps and brief

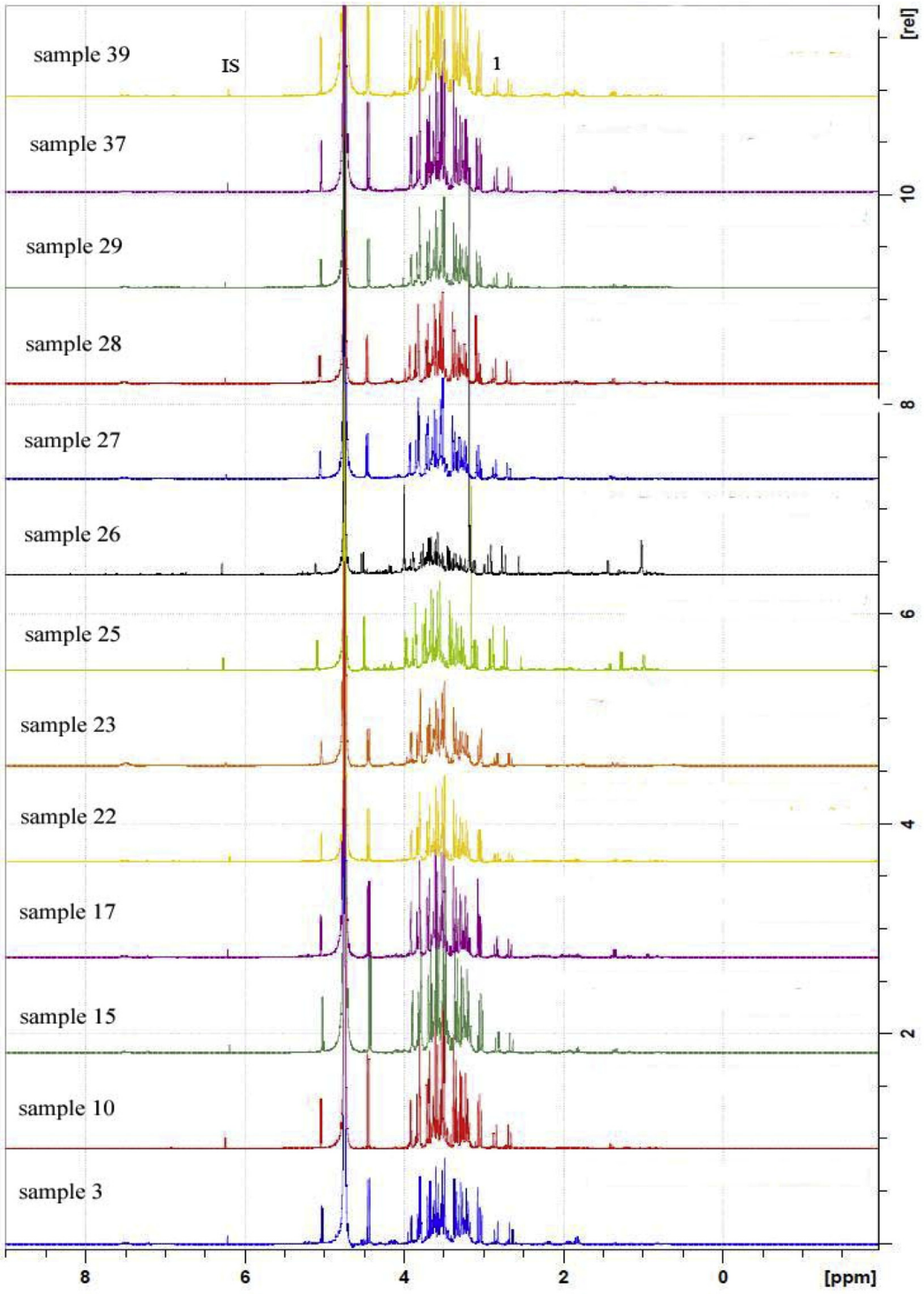

Fig. 3 – ^{1}H NMR spectra of LYW from samples 3, 10, 15, 17, 22–23, 25–29, 37, and 39. IS: maleic acid (δ 6.36, 2H). 1: citric acid (δ 2.81, 2H).

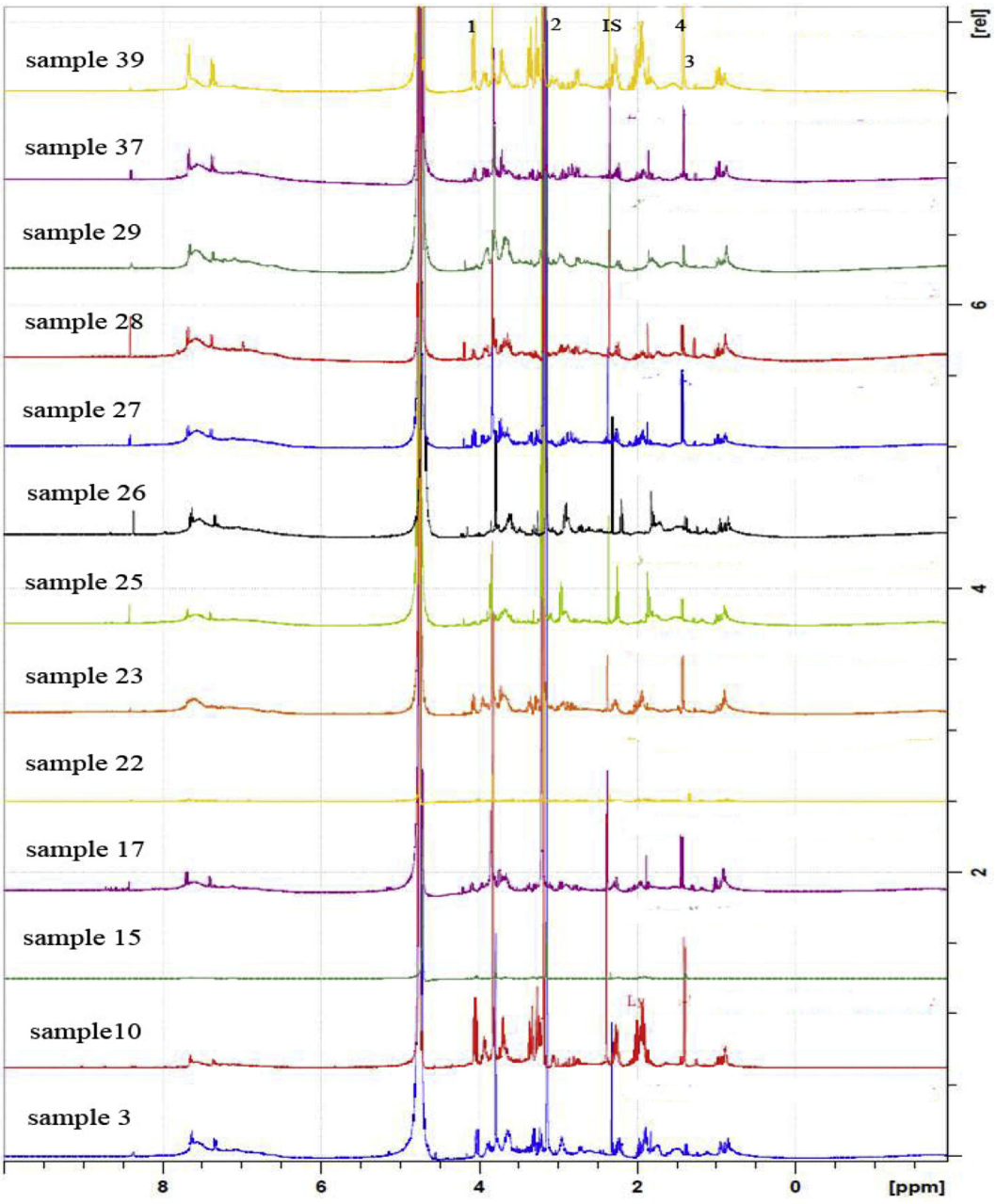

Fig. 4 – ^{1}H NMR spectra of LYN from samples 3, 10, 15, 17, 22–23, 25–29, 37, and 39. IS: succinic acid (δ 2.37, 4H); 1: proline (δ 4.08, 1H); 2: betaine (δ 3.21, 9H); 3: threonine (δ 1.28, 3H); 4: alanine (δ 1.43, 3H).

Table 2 – Contents of betaine, citric acid, threonine, alanine, and proline in samples 10 and 13 by conventional (adding internal standards) method and NMR Digital ERETIC.[a]

Samples no.	Betaine	Citric acid	Threonine	Alanine	Proline
10[c]	0.48 (0.50)[b]	1.14 (0.27)	0.02 (1.53)	0.10 (1.05)	0.46 (7.52)
13[c]	0.59 (0.36)	1.42 (0.09)	0.03 (6.10)	0.22 (1.11)	0.86 (0.76)
10[d]	0.48 (0.51)	1.07 (0.10)	0.02 (1.21)	0.10 (1.12)	0.46 (7.56)
13[d]	0.59 (0.34)	1.41 (0.21)	0.03 (6.53)	0.22 (1.08)	0.85 (0.86)

[a] Recorded on % (w/w) of *Lycium* species.
[b] % RSD, all experiments were based on triplicate measurement.
[c] Analysis by Digital ERETIC 2.
[d] Analysis by addition of internal stands.

experiment time also indicated this method as a feasible and practical tool for quality control and species authentication.

To demonstrate the practicality of the developed ^{1}H NMR method, 39 commercial *L. barbarum* products purchased from different places in China and 3 wild samples of *Lycium chinense* collected from Shanxi were analyzed using ^{1}H NMR, as reported in Table 1. Five grams of collected samples was extracted with water, passed through a strong cation exchanger SPE column and the cartridge was then washed with water. Maleic acid (0.5 mg) was added to the solution and the solvent was evaporated to dryness *in vacuo* to afford LYW fraction. 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 fraction. All samples were analyzed in triplicates to determine the contents of five target compounds, and the analytical ^{1}H NMR spectra of LYW and LYN from samples 3, 10, 15, 17, 22, 23, 25–29, 37 and 39 were shown in Figs. 3 and 4, respectively. The results obtained by the ^{1}H NMR method were found to be highly accurate and reproducible for determining the target compounds with relative standard deviations (RSD) $\leq$ 8.80%. The percentages of betaine of 42 *Lycium* fruit samples were determined within the range between 0.21 and 1.17% (Table 1). Among them, sample 35 contained the highest amount of betaine.

The advantages of the developed analytical method are discussed as follows. The SCX-SPE pre-processing method can collect charged compounds and separate out other interfering compounds. ^{1}H NMR can record compounds that are poorly detected by measuring UV absorbance, including betaine and amino acids. Moreover, ^{1}H NMR method provides a quantitative method of analyzing Lycii Fructus that does not require preparation of compound derivatives, control of pH values, or addition of NMR shifting reagents. In addition, several compounds can be simultaneously quantitated in a highly specific quality analysis. The analytical solvent system uses only water without any other organic solvents, which is very safe, convenient, uncomplicated, and green.

ERETIC 2 based on PULCON (Pulse Length-based Concentration determination) principle is a new fully automated concentration determination technology based on NMR without addition of any internal standards [23]. A known compound of interest is detected and defined as an ERETIC reference. The analysis is performed using the same parameters. Then the amounts of target compounds are determined by software processing. For example, samples 10 and 13 were analyzed by ERETIC 2 software and compared to the original method based on the addition of the internal standard to samples. The results for the contents of betaine, citric acid, proline, threonine, and alanine between the two methods were very similar (Table 2), indicating that the NMR ERETIC 2 method can be conveniently applied to the quantification of bioactive compounds in foods, plants, and medicines.

The developed ^{1}H NMR method was successfully applied for the rapid and reliable simultaneous determination of betaine, citric acid, threonine, alanine, and proline in various sources of *Lycium* fruits as well as those in commercial products. It offers a short analysis time and can serve as a useful tool for the routine analysis of betaine and four other compounds. Furthermore, application of the ERETIC 2 method is more conveniently for quality control. Therefore, the ERETIC 2 ^{1}H NMR method is highly applicable in the quantitative analysis of bioactive principles in the various samples of Lycii Fructus.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jfda.2018.01.001.

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