

The Uncertainty Assessment of Threonine Determination in Ginseng and Its Immune Activity

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Abstract: Ginseng is one of famous herbs, which has many medical functions, such as neuroprotective effects, and anticancer function, because ginseng contains many active substances, and threonine is one important ingredient. In this study, to establish a mathematical model of uncertainty assessment for Threonine content in Ginseng by the amino acid assay, the sources of uncertainty in the measurement process were completely concerned, the main sources of uncertainty were fully analyzed, and evaluated and calculated. The results showed, for 0.2083g sample, the Threonine determination in Ginseng showed a good linear relationship. IN conclusion, this method developed in this study is suitable for the Uncertainty factors assessment of threonine measurement in Ginseng by amino acid assay. We also did animal test. To detect the immune activity of threonine in Ginseng, we detected the CD3+, CD19+ using FACS of mice after oral administration.

Keywords: Ginseng, threonine, Immune Activity, CD3+.

1. INTRODUCTION

Ginseng is a powerful herb which can improve immunity because they contain many medicine ingredient and nutritional ingredient. Threonine is one of the important nutritional ingredients. For a long time, the main method for determination of threonine in Ginseng is amino acid assay, and the experimental procedures have been very developed. But the results of the same sample showed a certain difference, which because those experiments are performed by different labs or different people in same lab. We analyzed the causes of the difference through the determination of threonine in Genseng, evaluated the sources of this uncertainty of these quantitative results, and found that the quantification of threonine showed in a certain confidence interval, which play the role of the correction on the measurement of threonine.

2. MATERIALS AND METHODOLOGY

Ginseng samples were farmed in Jilin Province, China, dried at 60°C after mashing using organizations broken machine, filtered with 60 mesh sieve after crushing by pulverizer, and then mixed, separately stored in bottle for detection.

Threonine reference substance were purchased from the Beijing Academy of Agricultural Sciences, 14.62 mg/L, 6.0 mol/L hydrochloric acid solution, guarantee reagent, Beijing Chemical industry; water is pure water, Hangzhou Wahaha

Group Co., Ltd. MCI Buffer L-8500-PH Kit for Mitsubishi Chemical Corporation; Coloration liquid: R1, R2 is Japan, and by Wako Pure Chemical Industries Co., Ltd. All glassware and experimental apparatus are immersed by concentrated sulfuric acid, washed with deionized water.

0.1g of Ginseng powder were weighed by 0.1 mg precision balance accurately and placed in the hydrolysis tube added 10.00 ml 6.0 mol/L hydrochloric acid, N₂ flowed through for 1min, and covered with rubber plug. This hydrolysis tube was sealed after vacuumed by vacuum pump, placed in a 110°C thermostatic oven, hydrolyzed for 22 h. When the hydrolysis ended, hydrolysis solution was filtered and transferred to a 50.00 ml volumetric flask and add deionized water to a constant volume. 1.00 ml filtrate was put into the beaker, and evaporated to dryness in a vacuum dryer, and then 1-2 ml of water was dissolved and then evaporated to dryness and repeated 2 times, and finally the residue was dissolved with 1.00 ml of 0.02 mol/L hydrochloric acid, filtered with 0.22 μm polyethylene ether sulfone membrane filtration then for determination on the machine.

The temperature of the laboratory (20 ± 5) °C; volumetric flask, straw and other glass container are according to the JJG196-2006 test procedures [1] Class B equipment standards; 0.1mg division balance was used according to JJG 1036-2008 Verification regulation for Electronic balance [2] requirements; testing instruments (Agilent1200) meet JJG705-2002 test requirements for Verification regulation of liquid chromatographs [3]. Methanol and other reagents meet the analytical criteria, water was Wahaha water.

Japan's Hitachi L-8800 amino acid analyzer; electronic analytical balance: 1712mp8 oven thermostat: DG 30/14-II.

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Table 1. The experimental design.

Group	amount
control	25 mice
sinsenoside	25 mice
threonine	25 mice
Sinsenoside+threonine	25 mice

Immunizing dose and approaches

Chromatographic column: Elipses XDB C18 (4.6 × 250 mm, 4.6 μm); buffer: PH1, PH2, PH3 and PH4, pH 5; detection wavelength for reaction solution: R1, R2; 570nm (visible light); buffer flow rate was 4.0 ml/min, and the flow rate of reaction liquid was 4.0 ml/min; column temperature: 57°C; injection volume was 20 μl.

Immunity Activity Detection on Animal

100 Mice are divided into four groups as following (Table 1):

5mg sinsenoside, threonine and Sinsenoside+threonine respectively was given to one Bal/c mice by oral administration per day. 8 days later, half of each group mice for facs, and half of it were for lymphocyte transformation test.

Facs approaches:

1. The mice were killed and the put in 1% benzalkonium bromide for 3-5min.
2. Sterile gauze was prepared in sterile plates, and 2 ml PBS buffer was added into plates. The left lumbar region skin of mice was cut, spleen was taken out, put on gauze, and the spleen was minced into single cells.
3. The cells were transferred into centrifuge tubes, labeled, 1000 r/min for 10min, and discarded supernatant.
4. 500μl distilled water was added to precipitate, shaken gently, leaved there for 20s, removed red blood cell by cell disruption.
5. Appropriated amount PBS buffer was added into solution, in case the white cells break.
6. 2 min later, the supernatant was transferred into another tube, and labeled.
7. The cell solution was diluted into $1 \times 10^6 / 100\mu\text{l}$ for facs.

CD3⁺T, CD4⁺/CD8⁺ and CD19⁺ was detected μl FITC labeled antibody to mice CD3⁺ put into each tubes. PE labeled antibody to mice CD4⁺T, CD8⁺T, and CD19⁺, then 100μl cell supernatant solution ($10^6/\text{ml}$), incubated for 30min at room temperature (18°C-25°C), then detected by facs. 488nm wave length for the detection of FITC, 533 nm wave

length for the detection of PE, 10000 cells were detected, and analyzed by facs software.

3. RESULTS AND DISCUSSION

Preparation of standard curve: Take exactly 0.1, 0.2, 0.3, 0.4, 0.6 ml reference substance Radix angelicae dahuricae with a 1 ml single channel pipette. Get constant volume to 1ml and place in an automatic sampling bottle. According to above "chromatographic condition" to test peak area, using the least square method with the peak area A and Radix angelicae dahuricae standard concentration C (mg/ml) were for linear fit. The Radix angelicae dahuricae equation is:

$$A = 0.2088 + 44.9649C, r = 0.9999.$$

Determination of sample: Filtrate the sample solution with 0.22 μm nylon membrane, according to 2 "chromatographic condition", determine on the machine. The peak area of the sample area was measured, and the results were shown in Table 2.

Evaluation of Uncertainty

Mathematical model

Mathematical calculation formula for Threonine content measurement:

$$R = \frac{C_{\text{standard}} \times A_{\text{sample}} \times V_{\text{load}} \times V_{\text{volume}}}{A_{\text{standard}} \times m \times V_{\text{removal}} \times 10^6}$$

where R is the threonine content in the sample, %; C_{standard} means reference sample concentration, mg/L; V_{volume} is the final volume of the sample, mL; A_{sample} means peak area of a sample mAu; A_{standard} means the peak area of the reference substance, mAu; m is the sample weight, g; the volume of the sample V_{load} is the sample volume loaded on the machine, mL; V_{removal} is the sample volume removed.

The Major Source of Measurement Uncertainty

The method of Determination of Amino Acids in Foods according to GB/T 5009.124-2003A [4], there are the following mainly sources of uncertainty: (1) the uncertainty introduced by sample weight; (2) the uncertainty introduced by sample volume; (3) sample process introduces uncertainty; (4) the uncertainty introduced by the sample

Table 2. The content of threonine in different volume injection sample (n = 6).

Sample Amount, m(g)	Peak area	Result R, (%)	Mean, (%)	RSD, (%)
0.2083	469234	0.2968	0.2971	0.008656
0.2056	461412	0.2958		
0.2053	457122	0.2933		
0.2028	463257	0.3009		
0.2015	456055	0.2982		
0.2084	471341	0.2979		

peak area; (5) the uncertainty introduced by sample measurement repeat; (6) the uncertainty introduced by the non-linear of standard curve.

Evaluation of Measurement Uncertainty

The samples weighing introduced uncertainty [u(m)]:
The balance calibration introduced uncertainty: the balance test showed analytical balance error was 0.1 mg, according to rectangular distribution ($k = \sqrt{3}$), uncertainty component introduced by the balance calibration:

$$u(m) = \sqrt{2[(0.00005)^2 + (0.00005)^2]} = 0.0001g$$

Weighing variability introduced uncertainty: according to Uncertainty Evaluation Guide in chemical analysis [5], the analytical balance variability is about $0.5 \times$ the final significant figure, and the final significant figure of the analysis balance in our laboratory was 0.1 mg. So, weighing variability introduced the uncertainty components:

$$u_2(m) = 0.5 \times 0.1mg = 0.00005g$$

Weighing by the difference method should calculated uncertainty twice, so the uncertainty introduced by the weighing scales:

$$u(m) = \sqrt{2[(0.00005)^2 + (0.00005)^2]} = 0.0001g$$

The sample weighted $m = 0.2083g$, the relative standard uncertainty introduced by weighing:

$$u_{rel}(m) = 0.0001g / 0.2083g = 0.000480$$

The Final Volume of Samples Introduced Uncertainty [u(V)]

50 mL volumetric flask bring uncertainty: the uncertainty brought by the volume container includes the following four sources:

Uncertainty introduced by the calibration error: 50 mL A grade volumetric flask error allowed ± 0.05 mL [1] rectangular distribution ($k = \sqrt{3}$), 50 mL volumetric flask calibration introduced uncertainty:

$$u_1(V_{volume50}) = 0.05mL / \sqrt{3} = 0.0288mL$$

Repeatability: filled 10 times to 50 mL volumetric flask in one experiments, the standard deviation 0.02 mL can be directly as uncertainty, namely: $u_2(V_{50}) = 0.02$ mL.

Uncertainty introduced by the temperature: the temperature range is $\pm 5^\circ C$, in the manual, water expansion coefficient is 2.1×10^{-4} mL $^\circ C^{-1}$, then 50 mL volumetric flask volume change were:

$$\Delta V_{volume50} = 50 \times 2.1 \times 10^{-4} \times 5 = 0.0525mL$$

The confidence level of 0.95, contains the factor $K = 1.96$, the uncertainty introduced by the temperature change:

$$u_3(V_{volume50}) = 0.0525mL / 1.96 = 0.0267mL$$

Uncertainty components caused by reading: relative standard uncertainty introduced from 50 mL volumetric flask:

$$u_4(V_{volume50}) = 0.01 \times 50mL / \sqrt{6} = 0.204mL$$

The relative standard uncertainty brought by 50ml volumetric flask:

$$u_{rel}(V_{volume50}) = \sqrt{0.0288^2 + 0.02^2 + 0.0267^2 + 0.204^2} / 50mL = 0.00156$$

Simple Processing Introduced Uncertainty [u(rep)]

Repeatable measurement introduced uncertainty: the threonine content repeatable measurement results are shown in Table 3, the standard deviation obtained by Bessel formula:

$$S_x = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n-1}} = 0.000386\%$$

Repeatability uncertainty:

$$u_{rel}(rep) = u_{(rep)} / \bar{R} = \frac{0.000157\%}{0.2971\%} = 0.000528$$

$$u_{(rep)} = S_x / \sqrt{n} = 0.000386 / \sqrt{6} = 0.000157\%$$

Table 3. Peak area of threonine

Sample Injected Order	Peak Area	Mean	RSD%
1	469234	463070.1	0.01344
2	461412		
3	457122		
4	463257		
5	456055		
6	471341		

Sample Peak Area Measurement Introduced Uncertainty [u(AS)]

Generated Uncertainty of Peak Area by Repeated Measurements

Because there is only 6 data in the Table 2, so use range method for evaluation according to Errors Analysis and Measurement Uncertainty Evaluation [6], in which $n = 6$, $C = 2.53$.

$$u_A = \frac{R_{\text{range}}}{C_{\text{difference coefficient}}};$$

$$\text{Threonine: } u_{\text{rel}} = \frac{u_A}{A} = \frac{6041.897}{463070.1} = 0.0103$$

Instrumental Data Processing System Introduced Uncertainty

According to instrument manual and the general performance of integrator, so far, the maximum error of peak area convolution procedure by the liquid chromatography is 0.2% to 1%, then the peak area relative uncertainty

$$\text{components: } u_A = \frac{0.01}{\sqrt{3}} = 0.00577$$

Liquid chromatography used micro-injector for measurement, the injection uncertainty was 1%, then the

$$\text{relative uncertainty components: } u_{\text{injection}} = \frac{0.01}{\sqrt{3}} = 0.00577$$

The uncertainty introduced by data-processing system:

$$u_{\text{process}} = \sqrt{u_A^2 + u_{\text{load}}^2} = 0.0082 \quad (14)$$

So we can get the evaluation of the uncertainty introduced by sample peak area measurement:

$$u_{\text{rel}}(A_S) = \sqrt{u_{\text{process}}^2 + u_{\text{rel}}^2}$$

Threonine:

$$u_{\text{rel}}(A_S) = \sqrt{0.0103^2 + 0.0082^2}$$

$$= 0.0131\%; u_{\text{rel}}(A_S) = u_{(\text{rep})} / \bar{R} = \frac{0.0131\%}{0.2971\%} = 0.0440 \quad (16)$$

The nonlinear standard curve introduced uncertainty [u(line)]: The preparation concentration 0, 0.1, 0.2, 0.3, 0.4, 0.6 mL five threonine standard solution, measured twice for each concentration. According to the measurement data, using the least squares method to prepare standard working curve equation [6-8]: $A = 9130c - 0.002857$, correlation coefficient $r = 1$. The standard deviation of the standard curve equation was calculated, i.e. residual standard deviation. The peak area measured values by instruments were calculated according to the linear equation (in Table 4).

Standard curve residual standard deviation

$$S_R = \sqrt{\frac{\sum_{j=1}^n [A_{0j} - (a + bC_{0j})]^2}{n-2}} = 37.562 \text{ mg/L}$$

The uncertainty introduced by standard curve fitting:

$$u_{(\text{line})} = \frac{S_R}{b} \sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(\bar{C} - \bar{C}_0)^2}{\sum_{j=1}^n (C_{0j} - \bar{C}_0)^2}} = \frac{37.562}{9130}$$

$$\sqrt{\frac{1}{6} + \frac{1}{12} + \frac{(50.7160 - 26.4)^2}{2286.9}} = 0.00209 \text{ mg/L}$$

where S_R : Residual standard deviation of standard curve (residual standard deviation); b : Slope; p : Repeatable times for the measurement of samples; n : The points of standard curve; \bar{C} : The mean of the sample concentration; \bar{C}_0 : The mean of the each points concentration on standard curve; C_{0j} : The concentration of each standard solution.

So the uncertainty introduced by standard curve:

$$u_{\text{rel}}(\text{line}) = \frac{u_{(\text{line})}}{C} = \frac{0.00209 \text{ mg/L}}{50.7160 \text{ mg/L}} = 0.0000412$$

Composition Uncertainty, Expanded Uncertainty and their Results Expression

The above uncertainties are separated, so the composition relative uncertainty is:

Table 4. The results of residual calculation for standard curve

n	Concentration C _{0j} (mg/l)	Response Value A _{0j}	Calculated Value a+bC _{0j}	[A _{0j} -(a+bC _{0j})] ²	(C _{0j} - \bar{C}_0) ²	(C _{0j} - \bar{C}_0) ²
1	0	0 0	-0.002857	0.000008162 0.000008162	-26.4	696.96
2	9.9	90393.4 90393.6	90386.997	40.9984 43.5996	-16.5	272.25
3	19.8	180787.2 180787.3	180773.977	174.3192 176.9698	-6.6	43.56
4	29.6	271180.4 271180.6	271160.997	376.4764 384.2776	3.3	10.89
5	39.6	361574.2 361574.2	361547.997	686.5972 686.5972	13.2	174.24
6	59.4	542361 542361.4	542321.997	1521.2340 1552.5964	33	1089
	$\bar{C}_0 = 26.4$			$\sum 5643.6658$		$\sum 2286.9$

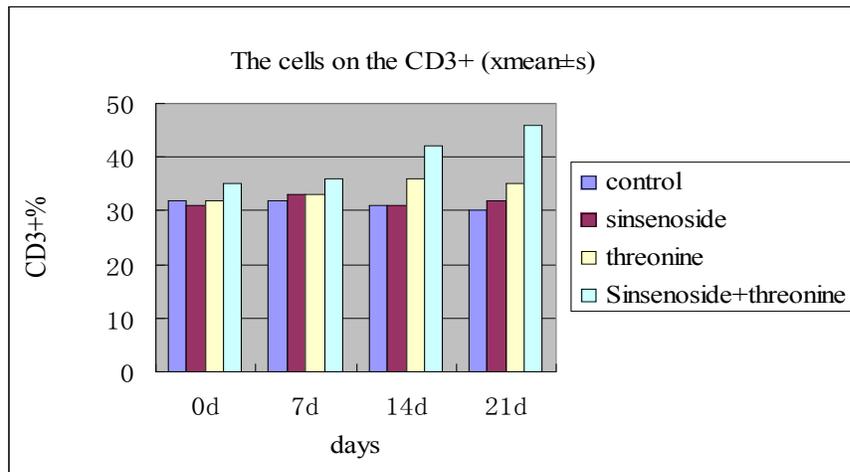


Fig. (1). Variation of CD3+T lymphocytes in spleen of mice after oral administration.

$$u_{rel}(R) = \sqrt{\frac{[u_{rel}(m)]^2 + [u_{rel}(V_{volume50})]^2}{+[u_{rel}(rep)]^2 + [u_{rel}(As)]^2 + [u_{rel}(line)]^2}}$$

$$= \sqrt{\frac{0.000480^2 + 0.00156^2 + 0.000528^2}{+0.0440^2 + 0.0000412^2}} = 0.0440$$

Composition uncertainty:

$$u(R) = u_{rel}(R) \times R = 0.0440 \times 0.2971\% = 0.0130\%$$

in which, inclusion factor $k = 2$; so the expended uncertainty:

$$U = ku(R) = 2 \times 0.0130\% = 0.0260\%$$

Finally, the content of threonine expressed as following:

$$R = (0.2971 \pm 0.0260)\%, k = 2$$

The Results of FACS

CD4+T, CD8+T, CD19+ cell subgroups were detected by FACS, and analyzed DBS (Figs. 1-3).

After oral administration, the three test groups compared to the control group, the increase of CD3+T, CD4+/CD8+, and CD19+ is significant ($P < 0.01$). The diversify of CD3+T, CD4+/CD8+, and CD19+ between groups

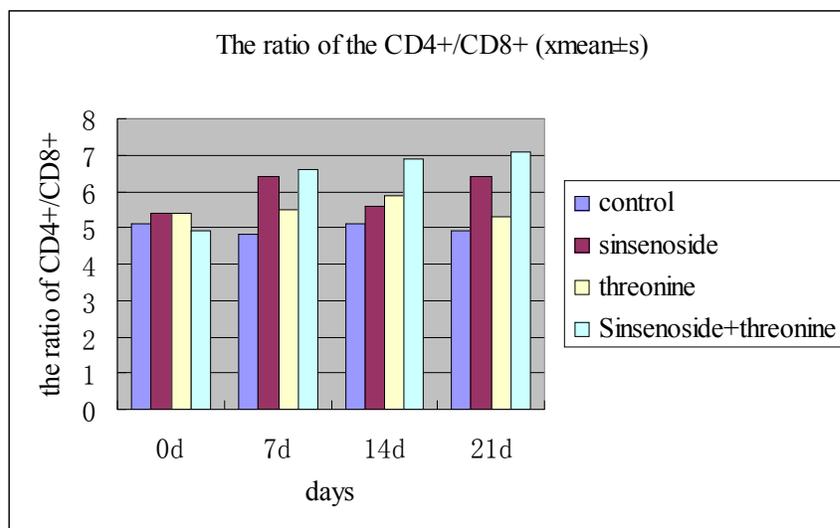


Fig. (2). Variation of C CD4⁺/CD8⁺ of lymphocytes in spleen of mice after oral administration.

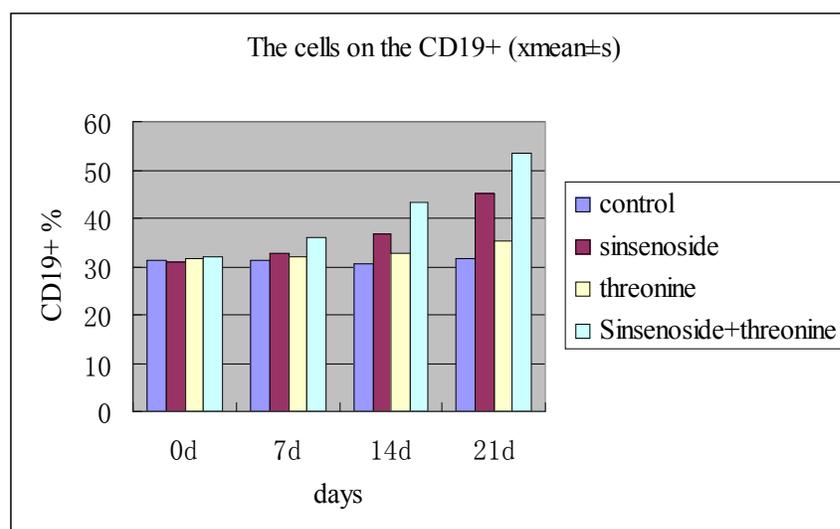


Fig. (3). Variation of CD19⁺T lymphocytes in spleen of mice after oral administration.

sinsenoside and threonine changed little ($P > 0.05$). Compared to control, sinsenoside and threonine group, the sinsenoside + threonine group, all tests change significantly ($P < 0.01$). So the threonine can improve the immunity, also this activity of threonine threonine in ginseng has synergism with ginsenoside.

CONCLUSION

From the whole evaluating process of the uncertainty, when threonine in Ginseng was determined by amino acids assay, whose uncertainty comes mainly from the sample peak area. So the uncertainty introduced by the sample peak area is the largest component. So for the determination of threonine in Ginseng, the control of the instrument sensitivity could reduce the uncertainty components. To reduce this component is the efficient way to reduce the measurement uncertainty. Also the threonine play an

important role on the enhancement of immunity, and this activity of threonine threonine in ginseng has synergism with ginsenoside. This study provides an effective analysis approaches for the identification of Ginseng, which is an important contribution for the application of Ginseng in Chinese Medicine.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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