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# Precise measurement for the purity of amino acid and peptide using quantitative nuclear magnetic resonance

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ABSTRACT

Precise measurement for the purity of organic compounds will fundamentally improve the capabilities and measurement services of the organic chemical analysis. Quantitative nuclear magnetic resonance (qNMR) is an important method to assess the purity of organic compounds. We presented a precise measurement method for the purity of small molecule with identification of impurities. In addition, the qNMR was rarely applied to purity of large compounds such as peptide, for which qNMR peaks are too crowded. Other than general idea of qNMR, we removed unwanted exchangeable peaks by proton exchange, as a new approach for qNMR, to make the quantitative protons of peptide isolated, which can ensure precise measurement. Moreover, a suitable internal standard, acesulfame potassium, was applied. The analytes were valine and peptide T5, due to their importance for protein analysis. For valine, the intraday CV was 0.052%, and the interday CV during 8 months was 0.071%. For peptide T5, simpler operation, shorter analytical time (1 h vs. 3 days) and smaller CV (0.36% vs. 0.93%) were achieved by qNMR, compared with a traditional method (amino acid based isotope labeled mass spectrometry) via a hydrolysis reaction. This method has greatly increased the quantitative precision of qNMR for small compounds, and extended application scope of qNMR from small compounds to peptides.



## 1. Introduction

Precise measurement for purity of organic compounds will fundamentally improve the capabilities and measurement services of the organic chemical analysis. Measurement precision is the closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions. It is usually expressed numerically by measures of imprecision, such as standard deviation, variance, or coefficient of variation under the specified conditions of measurement [1].

Generally speaking, the chemical purity of organic reference materials can be established according to one of the following approaches: (i) direct assay of the principal component; (ii) measurement of all detectable impurity components and subtracting these from 100%; or (iii) a combination of both approaches (i) and (ii). Methods commonly used for giving direct estimate of the principal component were gas chromatography–flame ionization detection, liquid chromatography–ultraviolet spectroscopy, quantitative nuclear magnetic resonance (qNMR), and elemental analysis. Methods commonly used for giving estimates of impurity components were differential scanning calorimetry, loss on drying at a specific temperature, Karl-Fisher titration, gas chromatography, nuclear magnetic resonance, ion chromatography, inductively coupled plasma-mass spectrometry, inductively coupled plasma-atomic emission spectrometry, X-ray fluorescence spectrometry and liquid chromatography–ultraviolet spectroscopy [2].

The approach (ii), also called the mass balance method, involves complicated experiments with various instruments [3,4]. The mass balance method needs to determine the total amount of the related structure organic substances, water, residual organic solvent and non-volatiles/inorganic substances by using various instruments. The purity value is 100% subtracting the total mass

*Abbreviations:* CRM, Certified reference material; CV, Coefficient of variation; DMSO, Dimethyl sulfoxide; hCG, Human chorionic gonadotropin; HPLC, High performance liquid chromatography; LC–MS/MS, Liquid chromatography–tandem mass spectrometry; NIM, National Institute of Metrology, China; NIST, National Institute of Standard and Technology, USA; NMR, Nuclear magnetic resonance; qNMR, Quantitative nuclear magnetic resonance; SRM, Standard Reference Material®; T5, The 5th peptide from the β-unit of hCG after tryptic-digestion

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fraction of impurities. For certification of the Standard Reference Material of amino acids in solution, National Institute of Standards and Technology of USA assessed the purity of 17 amino acids, by organic purity determination (liquid chromatography, titration, and thin layer chromatography), water content determination (Karl Fisher titration) and elemental analysis. The combined uncertainties of amino acids were 1–3% [5].

For the purity of peptide, the amino acid based isotope dilution mass spectrometry is a precise method. In this method, the purity of peptide is determined via hydrolysis of peptide into amino acids, with isotope labeled amino acids as internal standards. However, the hydrolysis reaction will increase uncertainty of measurement, and the coefficient of variance (CV) is about 0.8–1.5% [6–9].

The qNMR in approach (i) is a reliable quantitative spectroscopic technique in which the intensity of a resonance line is directly proportional to the number of resonant nuclei, so it can detect most of the organic compounds by using several certified reference materials as the internal standards. It is a promising primary and universal method with the following advantages. It is usually non-destructive and requires minimal sample preparation. It usually does not need to determine inorganic impurities (i.e. non-volatiles/inorganic substances and water). It can establish the traceability of the purity value for the analyte when the purity value of the internal standard can be traceable to the International Standard Units. However, before qNMR, one baseline-separated resonance for the analyte and one for the internal standard should be found. Also a careful identification of the analyte as well as its structure related organic impurities should be performed to confirm no overlapping on the peaks for quantification. QNMR was widely used in chemical purity assessments [2]. Often the solution state $^{1}$H-NMR with an internal standard method is used for purity assessment due to high sensitivity and high precision.

Malz and Jancke [10] studied the linearity, robustness, specificity, selectivity and accuracy of qNMR, and found that the maximum combined measurement uncertainty of round-robin tests is 1.5%. Pauli et al. [11] provided a quantitative $^{1}$H-NMR protocol with 13 key factors, including selection of NMR parameters, such as relaxation delay, pulse width, etc. Saito et al. [12] presented a practical guide for accurate quantitative solution state NMR analysis, and suggested that the accuracy is better than 1% if the key conditions are fulfilled. Saito et al. [13] determined the purity values of 17 organic pollutant using qNMR, and the variance (0.13%) is rather low among recent reports.

In this study, a precise qNMR measurement method for the purity of amino acid was presented. The relaxation delay, as a key parameter of qNMR, was optimized by experiments. Identification of impurities by LC–MS/MS facilitated the assignment of the impurity peaks.

In addition, the qNMR was rarely applied to determine the purity of large compounds such as peptide, because the qNMR peaks of peptide are too crowded to select an isolated peak for quantitative analysis. The general idea of qNMR is to select an isolated unexchangeable peak in the spectrum. Most quantitative NMR experiments avoid using the exchangeable protons due to the variation in the intensity of exchangeable proton which depends on the amount of deuterated solvent. Proton exchange, as a general technique for qualitative NMR, is rarely used in quantitative NMR. In this study, proton exchange was applied to remove unnecessary peaks (exchangeable proton) of peptide, in order to make quantitative peaks isolated, which can ensure precise measurement. After suppression of exchangeable protons by deuterium oxide, the isolated unexchangeable protons among exchangeable peaks were applied for quantification. Moreover, a suitable internal standard, acesulfame potassium, was selected.

The selected analytes were valine and peptide T5, due to their importance for protein analysis. Absolute quantification of protein is often undertaken by hydrolysis or enzymatic digestion. In analysis via hydrolysis, the traceability of protein is based on the purity of the amino acids. In analysis via digestion (often using trypsin), the traceability of protein is based on the purity of its specific peptides. Precise assessments of the purity of amino acids and specific peptides can greatly decrease the measurement uncertainties and improve the accuracy of protein. Valine is an important amino acid, which is often used as the reference material for protein [7–9]. Human chorionic gonadotropin (hCG) is an important glycoprotein hormone protein which is monitored in pregnancy testing, cancer detection and doping control in sports [14]. The peptide T5 (sequence: VLQGVLPALPQVVCNYR, molecular weight: 1869 g mol$^{-1}$, structure: see Fig. 1) is the 5th peptide from the β-unit of hCG after tryptic-digestion. The peptide T5 is a specific marker for hCG determination [15,16]. For establishment of metrological traceability, all candidates as internal standards were reference materials certified by National Institutes of Metrology. The aim of this study is to develop a precise qNMR method for the certification of the purity reference materials of amino acids and peptides, which can underpin the absolute quantification of protein.

## 2. Experimental

### 2.1. Materials and reagents

Valine was the sample of the intercomparison study (CCQM-K55.c) organized by Bureau International des Poids et Mesures (BIPM). For comparison, the valine purity CRM (certified reference material, No. GBW(E)100055) of NIM (National Institute of Metrology, China) with a purity value of 99.4% and an expanded uncertainty of 1.4% (coverage factor $k=2$) was also used. Creatinine was the SRM (Standard Reference Material) 914a from NIST (National Institute of Standard and Technology, US), with a purity value of 99.7% and an expanded uncertainty of 0.3% ($k=2$). Benzoic acid was the SRM 350b of NIST, with a purity value of 99.9978% and an expanded uncertainty of 0.0044% ($k=2$). Ethyl paraben was the CRM (No. GBW(E)100064) of NIM, with a purity value of 99.7% and an expanded uncertainty of 0.2% ($k=2$). Acesulfame potassium was

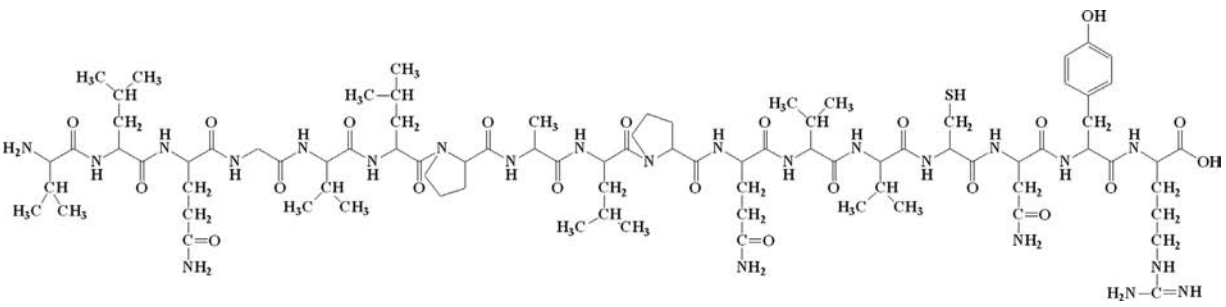

**Fig. 1.** Structure of peptide T5.

the CRM (No. GBW100065) of NIM with a purity value of 99.6% and an expanded uncertainty of 0.6% ($k=2$). Deuterium oxide ($D_2O$) and dimethyl sulfoxide-$d_6$ (DMSO-$d_6$) were purchased from Sigma-Aldrich (US). Alanine, leucine and isoleucine were the CRMs of NIM. 2-Aminobutyric acid was purchased from TCI (Tokyo, Japan). Peptide T5 was purchased from GL Biochem (Shanghai, China). $^{13}C_5$-proline(98%) and $d_{10}$-leucine (98%) were purchased from Cambridge Isotope Laboratories (MA, USA).

### 2.2. Apparatus

Main measurements were carried out on a Bruker Ascend 800 spectrometer with a 5 mm CPQCI cryoprobe at 800 MHz ($^1H$). The TopSpin 3.1 Bruker NMR software was used for data processing. The weighing was carried out on a Sartorius SE 2 balance ($d=0.1$ μg). Identification of impurities was performed on a Shimadzu HPLC–IT–TOF–MS (high performance liquid chromatography–ion trap–time of flight–mass spectrometry).

### 2.3. Experiment and calculation of qNMR

The sample solution of valine was prepared by following steps: valine (10–20 mg) and creatinine (5–8 mg) were accurately weighed, dissolved in $D_2O$ (1 mL), and transferred to a NMR tube. The sample solution of peptide T5 was prepared by following steps: peptide T5 (2–2.5 mg) and acesulfame potassium (0.8–1 mg) were accurately weighed, dissolved in DMSO-$d_6$ (0.500 mL) and $D_2O$ (0–0.050 mL), and transferred to an NMR tube.

The experiments were carried out using the following parameters optimized for qNMR: 30° pulse, 65,536 data points, relaxation delay of 32 s for valine and 36 s for peptide T5, and 32 scans. Fourier transformation was done with exponential filtering of zero after zero filling the data to 65,536 time domain points.

The calculation equation of qNMR [2,13] for the purity is as follows:

$$P_x = \frac{I_x}{I_s}\frac{N_s}{N_x}\frac{M_x}{M_s}\frac{m_s}{m_x}P_s \tag{1}$$

where $I_s$, $N_s$, $M_s$, $m_s$ and $P_s$ are the peak area, number of proton, molecular weight, mass and purity of the internal standard, respectively. $I_x$, $N_x$, $M_x$, $m_x$ and $P_x$ are the peak area, number of proton, molecular weight, mass and purity of the sample, respectively.

Since the number of proton in a molecule is certain, the relative standard uncertainty of assign value is

$$\frac{u(P_x)}{P_x} = \sqrt{\left(\frac{u(I_x/I_s)}{I_x/I_s}\right)^2 + \left(\frac{u(M_x)}{M_x}\right)^2 + \left(\frac{u(M_s)}{M_s}\right)^2 + \left(\frac{u(m_x)}{m_x}\right)^2 + \left(\frac{u(m_s)}{m_s}\right)^2 + \left(\frac{u(P_s)}{P_s}\right)} \tag{2}$$

In experiments using advanced balances, the uncertainty from mass is often neglectable. The uncertainty from molecular weight is often rather small. Therefore, the main contribution of the uncertainty was often from the first polynomial (repeatability of peak area) and the last polynomial (purity of the internal standard).

### 2.4. LC–MS/MS

In LC–MS/MS for valine, aliquots (2 μL) of calibrators or samples were separated by HPLC on a Primesep 100 column (2.1 × 250 mm, 5 μm) at 25 °C with a mobile phase consisting of 80% water (with 0.5% formic acid) and 20% acetonitrile (with 0.5% formic acid). The flow rate was 0.4 mL $min^{-1}$.

### 2.5. Amino acid based isotope dilution mass spectrometry for peptide T5

The experiments were in accordance with reference [6] for hydrolysis and amino acid based isotope dilution mass spectrometry for peptide T5. Before hydrolysis, specific amount of isotope labeled leucine and proline ($d_{10}$-leucine and $^{13}C_5$-proline) were added as internal standards, to make the mass ratios of unlabeled/labeled amino acids about 1:1. For calibration, a higher level standard solution and a lower level standard solution were prepared in the mass ratio of 1.1:1 and 0.9:1, respectively. After hydrolysis, leucine and proline were determined by mass spectrometry. The purity result of peptide T5 was the mean of results calculated from the two amino acids.

The equation for concentration of leucine ($c_{Leu}$) after hydrolysis [6] is

$$c_{Leu} = \frac{PP_H m_s[R_{sample}(I_1 - I_2) - (I_1R_2 - I_2R_1)]}{m(R_1 - R_2)} \tag{3}$$

where $P$ is the purity of leucine, $P_H$ is hydrolysis efficiency of peptide T5, $m_s$ is the mass of $d_{10}$-leucine, $R_{sample}$ is the leucine/$d_{10}$-leucine area ratio of the sample solution, $I_1$ is the leucine/$d_{10}$-leucine mass ratio of the lower level standard solution, $I_2$ is the leucine/$d_{10}$-leucine mass ratio of the higher level standard, $R_1$ is the leucine/$d_{10}$-leucine area ratio of the lower level standard, $R_2$ is the leucine/$d_{10}$-leucine area ratio of the higher level standard, and $m$ is the sample mass.

The purity of peptide T5 calculated from leucine ($P_{Pep,Leu}$) is

$$P_{Pep,Leu} = \frac{C_{Leu}MPep}{N_{Leu}M_{Leu}} \tag{4}$$

where $M_{Pep}$ is the molecular weight of peptide T5, $M_{Leu}$ is the molecular weight of leucine, and $N_{Leu}$ is the number of leucine residue in the peptide T5.

The concentration of proline after hydrolysis ($c_{Pro}$) and the purity of peptide T5 calculated from proline ($P_{Pep,Pro}$) were obtained by similar equations as Eqs. (3) and (4).Then, the purity result of peptide T5 was calculated as follows:

$$P_{Pep} = \frac{P_{Pep,Leu} + P_{Pep,Pro}}{2} \tag{5}$$

### 2.6. Comparison of the results

For comparison of the result with the certified value [17], absolute difference ($\Delta$) between the mean measured value ($c_m$) and the certified value ($c_{CRM}$) was calculated (Eq. 6). The uncertainty of $\Delta$ was also calculated (Eq. 7). To evaluate method performance, $\Delta$ is compared with $U_\Delta$; if $\Delta \le U_\Delta$, then there is no significant difference between the measurement result and the certified value [17]

$$\Delta = |c_m - c_{CRM}| \tag{6}$$

$$U_\Delta = 2\sqrt{u_m^2 + u_{CRM}^2} \tag{7}$$

The $F$-test was applied to test equal-precision between the two different methods, in which equal-precision requires

$$f_\alpha(\nu_1,\nu_2) < F < \frac{1}{f_\alpha(\nu_1,\nu_2)} \tag{8}$$

$$F = \frac{S_1^2}{S_2^2} \tag{9}$$

where $S_1$ and $S_2$ are the standard deviations of two methods, $\alpha$ is the degree of confidence, and $\nu_1$ and $\nu_2$ are the degrees of freedom of two methods.

The *t*-test was applied to test consistency between two methods, in which consistency requires

$$|t| < t_{a,(n_1+n_2-2)} \tag{10}$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{((n_1-1)S_1^2+(n_2-1)S_2^2)}{(n_1+n_2-2)} \bullet \frac{(n_1+n_2)}{n_1 n_2}}} \tag{11}$$

where $n_1$ and $n_2$ are the numbers of determination of the two methods.

## 3. Results and discussion

### 3.1. Selection of internal standard for valine

Benzoic acid is a SRM with high purity and very small uncertainty, but its solubility in water is low (~1.8 mg/mL). Water, methanol, dimethyl sulfoxide, as well as mixture solvent of methanol and water were examined. No suitable solvent can dissolve both benzoic acid and valine with sufficiently high concentrations. Both creatinine and valine can be dissolved in water with sufficiently high concentrations. Solubility of valine in water is more than 43 mg/mL. Moreover, the NMR peak of creatinine with the chemical shift ($\delta$) of 2.9 does not overlap with any peak of valine. Therefore, creatinine was selected as the internal standard.

### 3.2. Assignment of peaks for valine

According to the result of LC–MS (Fig. 2), by comparison with NMR spectra of authentic samples of each impurity (Fig. 3), and assisted by a 2D-$^1$H-COSY (correlation spectroscopy) (Fig. 4), four impurities in the valine sample (alanine, leucine, isoleucine and 2-aminobutyric acid) were identified. All peaks in the valine sample were identified, as shown in Fig. 5.

### 3.3. Selection of quantitative peak for valine

Based on the outcome of this assessment, the valine beta proton at $\delta$=2.1 and the creatinine N–$CH_3$ singlet at $\delta$=2.9 were selected as the peaks for quantification. Because the $^{13}$C satellite peaks of the valine beta proton at $\delta$=2.1 did not resolve with their major peak, the integration of these peaks included respective $^{13}$C satellite peaks. Moreover, in order to confirm no overlapping

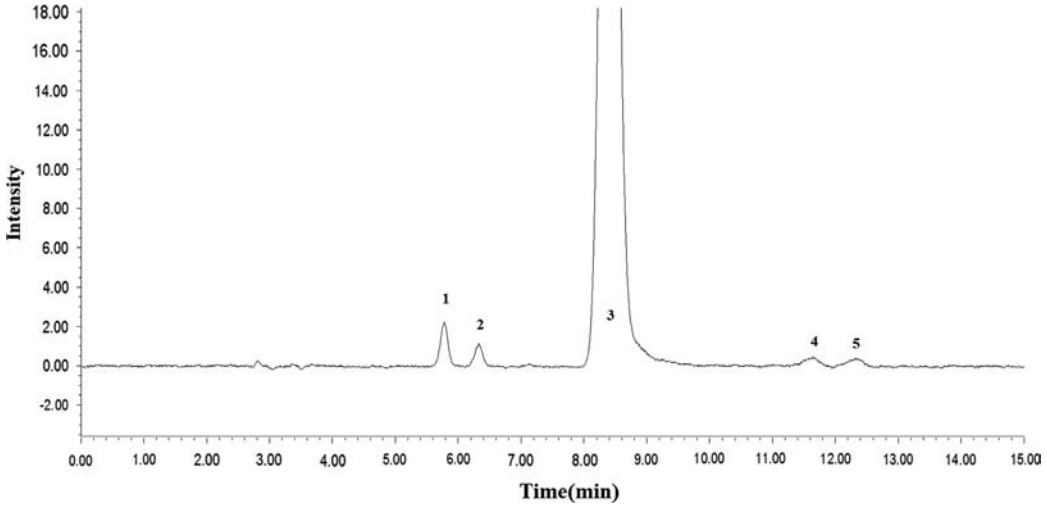


**Fig. 2.** The chromatogram of valine sample by LC–MS. 1: unknown peak from blank solvent, 2: alanine, 3: valine, 4: isoleucine, and 5: leucine.

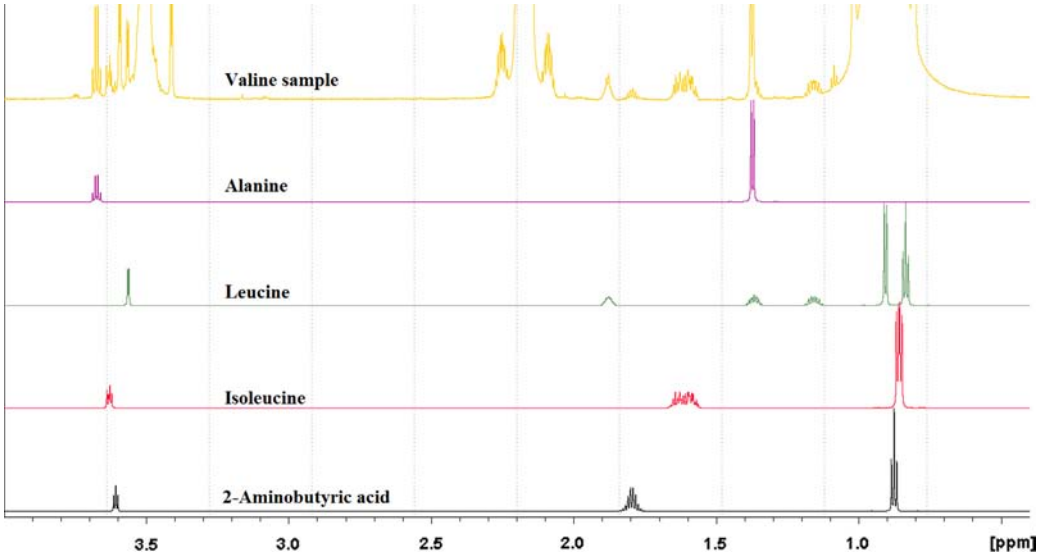


**Fig. 3.** Comparison with NMR spectra of the valine sample and authentic samples of each impurity.

between the two peaks for quantification, creatinine/$D_2O$ solution and valine/$D_2O$ solution were separately determined by NMR. No visible peak was observed at $\delta=2.9$ in the valine solution, and no visible peak was observed at $\delta=2.1$ in the creatinine solution.

### 3.4. Pulse angle and relaxation delay for valine

Longer pulse angle can obtain higher sensitivity, but longer relaxation delay is needed; thus pulse angle of 30° was applied. Relaxation delay ($d_1$) is a key parameter of qNMR, which is suggested to be more than $5T_{1,max}$ in qNMR. The respective spin-lattice relaxation time values ($T_1$) for the two quantitative peaks were determined, which were 2.6 s (peak of $\delta=2.9$) and 2.4 s (peak of $\delta=2.1$). Thus the largest spin-lattice relaxation time ($T_{1,max}$) for this quantification was 2.6 s. Relaxation delay values of 8 s, 16 s, 32 s and 64 s were examined for a sample solution. The resulting ±CVs were 99.720 ± 0.050%, 99.265 ± 0.038%, 99.013 ± 0.026% and 99.006 ± 0.021%, correspondingly. The difference between results of 32 s and 64 s was 0.007%, which was less than respective CVs. Therefore, relaxation delay of 32 s (or 12.3 $T_{1,max}$) was applied to achieve sufficient precision in reasonable experiment time.

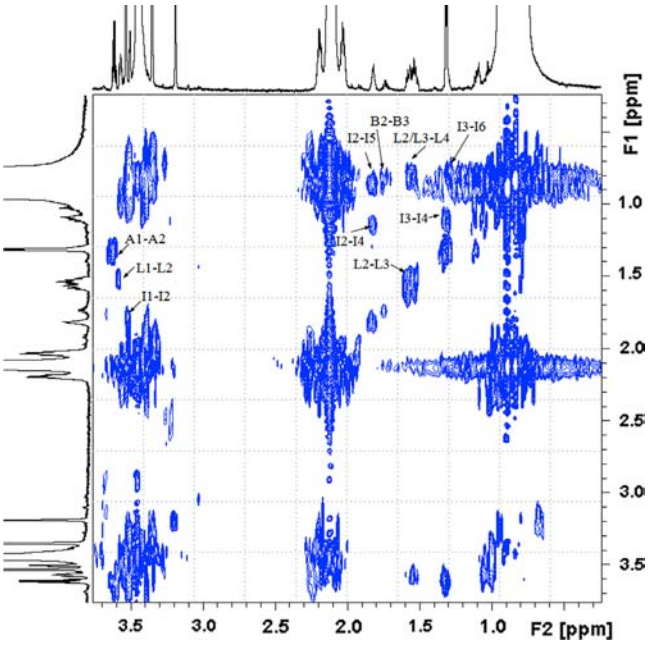


**Fig. 4.** 2D-$^1$H-COSY of the valine sample and correlation peaks of impurities. For the labels of peaks, see Fig. 5.

### 3.5. Linearity of valine

For linearity test, the mass ratio of valine/creatinine ($x$) varied from 1:10 to 10:1 in 6 sample solutions, and the peak area ratios of valine/creatinine ($y$) were determined. Since this study is for the purity of a compound, not an analyte in a matrix, the concentration was designed based on three considerations: (1) since too low concentration ( < 1 mg/mL) leads to low sensitivity, concentration was almost higher than 1 mg/mL; (2) since too high concentration may lead to insolublity of sample, all concentrations of valine were less than 25 mg/mL and those of creatinine were less than 10 mg/mL; (3) the mass ratio of valine to creatinine varied from 0.1 to 10. As shown in Table 1, the concentration range of valine was 1–25 mg/mL and that of creatinine was 1–10 mg/mL. The quantification concentration (valine: 10–20 mg/mL; creatinine: 5–8 mg/mL) was covered by this linearity test. The data and regression line are shown in Fig. 6. The regression formula was $y=0.32017x$, and the regression coefficient ($r$) was 0.99998.

### 3.6. Precision of results for valine

For intraday test, the results of seven sample solutions within one day are shown in Table 2, and the CV was 0.052%. For interday test, various samples were prepared and measured at various days

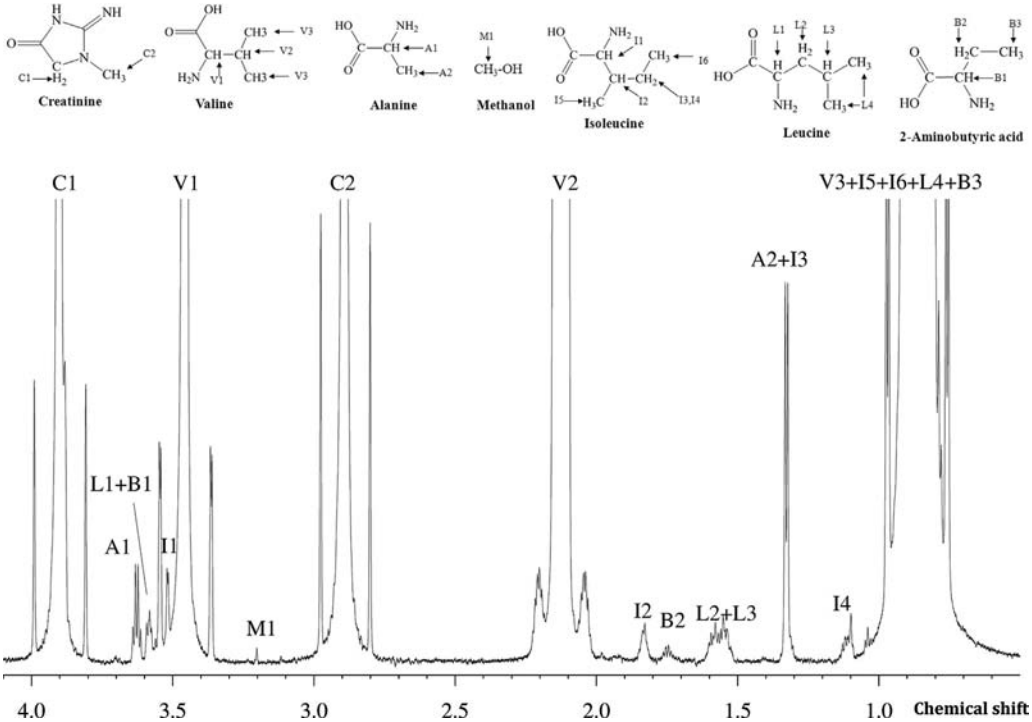


**Fig. 5.** The 800 MHz $^1$H-NMR spectrum of sample solution (valine, creatinine and $D_2O$) and the structures of creatinine, valine and impurities in the valine sample. The labeled peaks were assigned to protons in the molecules.

**Table 1**
Results of linearity test.

| $C_{Val}$ (mg/mL) | $C_{cre}$ (mg/mL) | $R_m$ | $R_A$ |
|---|---|---|---|
| 1.1656 | 9.9154 | 0.1176 | 0.0376 |
| 16.8348 | 8.3868 | 2.0073 | 0.6393 |
| 24.8040 | 6.0103 | 4.1269 | 1.3260 |
| 24.9920 | 4.1423 | 6.0333 | 1.9366 |
| 16.4023 | 2.0933 | 7.8355 | 2.5186 |
| 10.1763 | 0.9681 | 10.5116 | 3.3517 |

$C_{val}$: the concentration of valine, $C_{cre}$: the concentration of creatinine, $R_m$: the mass ratio of valine/creatine in the solution, and $R_A$: the peak area ratio of valine/creatine in qNMR.

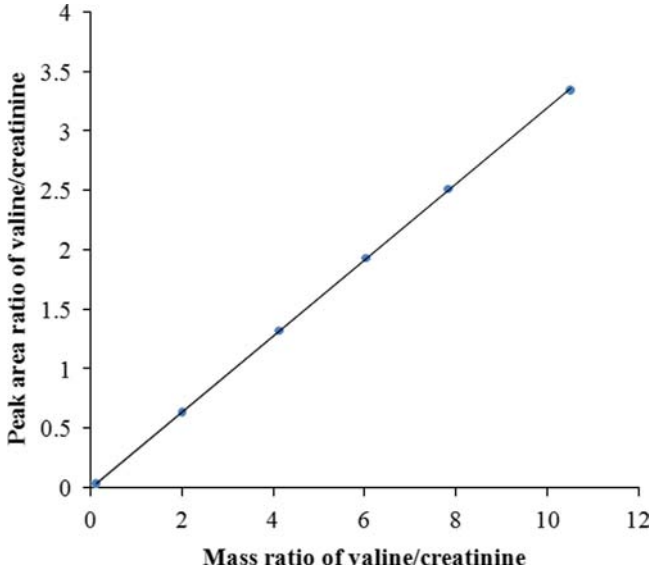


**Fig. 6.** Linearity study for valine with creatinine as an internal standard.

**Table 2**
The intraday and interday tests for purity of valine (g $g^{-1}$)

| Intraday test | | Interday test | |
|---|---|---|---|
| Sample | Purity (%) | Day (number of replicate sample) | Purity (%) |
| 1 | 99.132 | 0 ($n$=3) | 98.973 |
| 2 | 99.141 | 1 ($n$=3) | 98.937 |
| 3 | 99.220 | 15 ($n$=2) | 98.955 |
| 4 | 99.060 | 30 ($n$=7) | 99.134 |
| 5 | 99.170 | 60 ($n$=6) | 99.012 |
| 6 | 99.117 | 250 ($n$=6) | 99.013 |
| 7 | 99.100 | | |
| Mean | 99.134 | Mean | 99.004 |
| CV | 0.052 | CV | 0.071 |

in 8 months (250 days), and the result of each day was a mean result from 2 to 7 samples at that day. The interday CV was 0.071% (Table 2). The high precision was mainly contributed by high signal-to-noise ratio, which was 4179 for the valine beta proton (10 mg/mL).

### 3.7. Comparison with the traditional method for valine

The purity CRM was developed by NIM using a mass balance method with liquid chromatography and titration. The certified value was 99.4% with a combined uncertainty of 0.8%. Seven portions of the CRM were determined by this qNMR method, and the results were 99.751%, 99.724%, 99.859%, 99.598%, 99.712%, 99.767% and 99.691% (mean=99.729%, standard deviation=0.079%, and combined uncertainty=0.17%). According to Eqs. (6) and (7), absolute difference ($\Delta$) and its uncertainty ($U_\Delta$) were calculated, and were compared

$$\Delta = |c_m - c_{CRM}| = |99.729\% - 99.4\%| = 0.33\%$$

$$U_\Delta = 2\sqrt{u_m^2 + u_{CRM}^2} = 2\sqrt{0.17\%^2 + 0.8\%^2} = 1.6\%$$

Since $\Delta \le U_\Delta$, the qNMR results are consistent with the certified value. Moreover, the CRM may be recertified by this qNMR method.

### 3.8. Assignment of peaks for peptide T5

The spectra of TOCSY (total correlation spectroscopy) and NOESY (nuclear Overhauser effect spectroscopy) of peptide T5 dissolved in DMSO-$d_6$ are shown in Fig. 7. The peaks for all protons of peptide T5 were assigned. In the fingerprint area, all TOCSY peaks were identified as correlation between the α-CH and the backbone NH of each amino acid residues (marked in Fig. 7 as 1–6, 8 and 9 and 11–17). Since proline residue had no backbone NH, no TOCSY peak existed in that area; the NOESY peak between the α-CH of a proline residue and the backbone NH of its next residue is marked in Fig. 7 (7 and 10). Since arginine had an –NH– group in the side chain, the TOCSY peak between the proton of this group and it neighboring $\delta$-CH was found (marked as 17').

In the 16th residue, 2,6H and 3,5H represented the protons in the phenol group of the tyrosine residue. These two peaks were correlated with each other in TOCSY, and were also correlated with β-$CH_2$ of the 16th residue in NOESY. As shown in TOCSY spectrum, these two peaks were not overlapped with the other peaks in the $^1$H-NMR. The $^1$H-NMR spectrum is shown in Fig. 8(a).

### 3.9. Selection of internal standard for peptide T5

Since a peptide is a polymer of amino acids, the spectrum is characteristic. In general, chemical shifts of $NH_x$ protons and $CH_x$ protons are 6.5–8.5 and 0.5–4.6, respectively. Therefore, it is preferable to spike an internal standard which has any proton in chemical shift of 4.6–6.5. Acesulfame potassium was selected as an internal standard, which has a proton with chemical shift of 5.3. Moreover, it is non-volatile and non-hygroscopic, so it is easy to be weighed accurately.

### 3.10. Selection of quantitative peak for peptide T5

It is difficult to select an isolated NMR peak for complex molecules. As shown in Fig. 8(a), the protons at 0.5–4.6 are too crowded, so no suitable peak can be selected. Although some NH protons at 6.5–8.5 are isolated, the NH protons are exchangeable protons. Based on the principle of NMR, the intensity of exchangeable proton is unsuitable for quantification. However, the 2,6H peak ($\delta$=7.0) and 3,5H ($\delta$=6.6) peak are CH protons in the phenol group of tyrosine residue. They are not exchangeable proton, and they locate among some NH protons. By spiking deuterium oxide ($D_2O$), the exchangeable protons will exchange with the deuterium oxide, and the peaks of exchangeable protons will decrease or disappear in the NMR spectrum. Therefore, it is a good mean to make the two phenol peaks isolated.

### 3.11. Relaxation delay for peptide T5

The relaxation time values ($T_1$) for the three quantitative peaks were determined, which were 2.5 s (peak of $\delta$=7.0), 3.8 s (peak of $\delta$=6.7) and 7.2 s (peak of $\delta$=5.3). According to the experiments, relaxation delay of 36 s (or $5T_{1,max}$) was applied to achieve sufficient precision in reasonable experiment time.

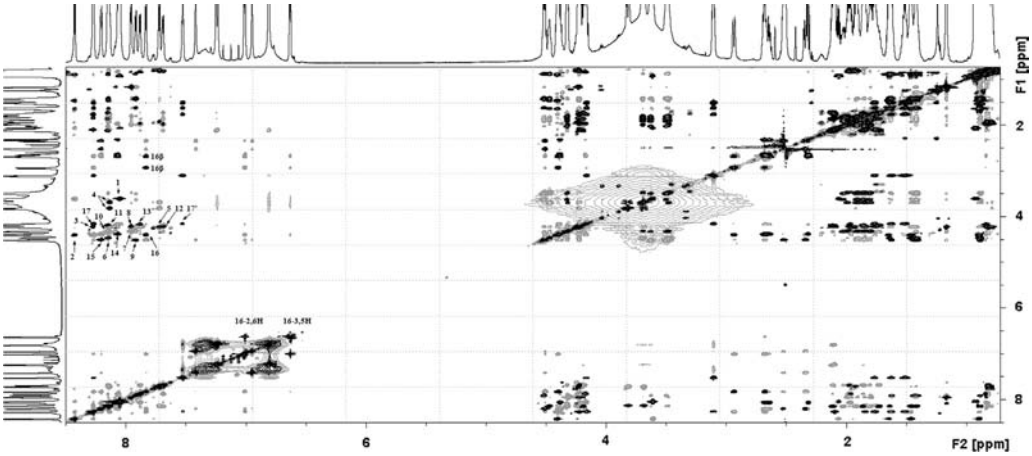


**Fig. 7.** TOCSY (black) and NOESY (gray) of peptide T5 dissolved in DMSO-$d_6$, 1–6, 8 and 9 and 11–17: the TOCSY correlation between the α-CH and the backbone NH of each amino acid residues, 7: the NOESY correlation between the α-CH of 7th residue (proline) and the backbone NH of 8th residue, 10: the NOESY correlation between the α-CH of 10th residue (proline) and the backbone NH of 11th residue, 17': the TOCSY correlation between the δ-CH and its neighboring NH (the underlined: $-CH_2-CH_2-CH_2-NH-C(=NH)-NH_2$.) in the 17th residue (argenine), 16β: the TOCSY correlation between the β-$CH_2$ and the backbone NH of 16th residue (tyrosine), 16: 2,6H–16:3,5H: the TOCSY correlation between the 2,6H and the 3,5H of the 16th residue.

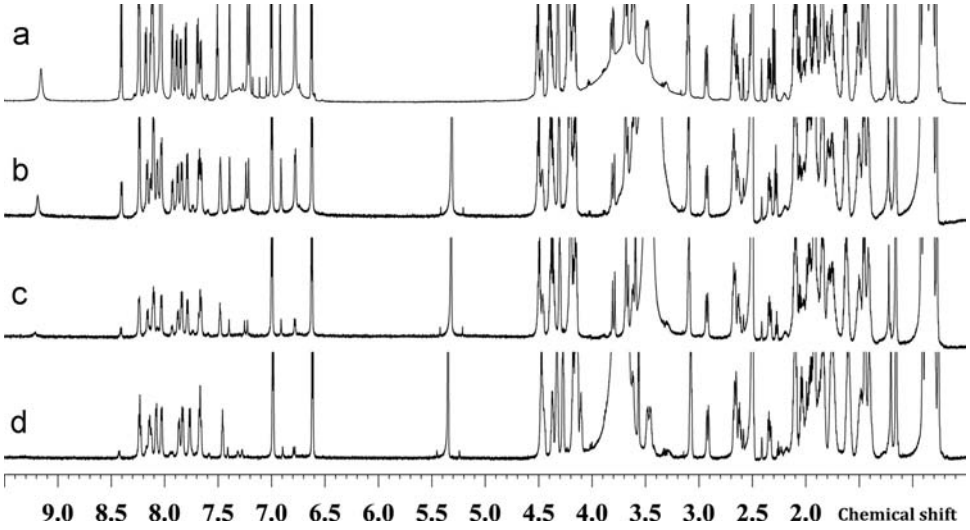


**Fig. 8.** The $^1$H-NMR spectra of the sample solutions of 2.4 mg peptide T5 (sequence VLQGVLPALPQVVCNYR) and 0.5 mL DMSO-$d_6$. (a) No spiking, (b) acesulfame potassium (1 mg) and $D_2O$ (0.005 mL) spiked, (c) acesulfame potassium (1 mg) and $D_2O$ (0.015 mL) spiked, and (d) acesulfame potassium (1 mg) and $D_2O$ (0.050 mL) spiked. Numbers of scan were 64 (a) and 32 (b–d).

### 3.12. Precision of results for peptide T5

Sample solutions of peptide T5 with various volume ratios of $D_2O$/DMSO-$d_6$ (1:100, 3:100 and 10:100) were prepared, and the NMR spectra are shown in Fig. 8(b–d). Compared with Fig. 8(a), $D_2O$ had made the two phenol peaks isolated. At each ratio, the CVs by using quantitative peak of $\delta=7.0$ were less than those of $\delta=6.6$. So the peak of $\delta=7.0$ was used for quantification. The result of sample solutions with three ratios (mean=83.09%, CV=0.48%) was consistent with the result within the ratio of 1:100 (the sample 1–3 of qNMR column is shown in Table 3, mean=83.09%, CV=0.49%). It indicated that 1% $D_2O$ is sufficient for accurate quantification.

The signal-to-noise ratio of peptide T5 ($\delta=7.0$) was 378. Since its molecular weight (1869 g $mol^{-1}$) is larger than valine (117 g $mol^{-1}$), its molar concentration (2 mmol $L^{-1}$) is much less than that of valine solution (86–171 mmol $L^{-1}$). Therefore, the CV of peptide T5 is larger than that of valine. In order to improve the precision, three peptide T5 solutions (12 mmol $L^{-1}$) for qNMR were prepared by weighing 12 mg of peptide T5, 6 mg of acesulfame potassium, 0.050 mL of $D_2O$, and 0.5 mL of DMSO-$d_6$. The signal-to-noise ratio of peptide T5 ($\delta=7.0$) was enhanced to 1224, and the CVs from $\delta=7.0$ and $\delta=6.6$ were 0.30% and 0.21%, respectively. However, this experiment spent more amount of peptide (12 mg per test), so 2 mmol $L^{-1}$ of peptide solution was recommended.

### 3.13. Comparison with the traditional method for determination of peptide T5

Seven portions of peptide T5 were measured by amino acid based isotope labeled mass spectrometry, and the results are shown in Table 3. The *t*-test result agreed, which indicated that the results between the two methods were consistent. But the *F*-test result did not agree, which indicated that the precision between the two methods was not equal. The CV (0.93%) of the traditional method was rather good, compared with related literatures (0.8–1.5%) [6–9], but it was larger than the CV of qNMR (0.36%), since a hydrolysis reaction was needed in the amino acid base method. In contrast, the qNMR method is a direct spectroscopic assessment without any reaction, and its precision can be enhanced by increasing the number of scans (the signal-to-noise

**Table 3**
The purity results of peptide T5 by qNMR and the traditional method (amino acid based isotope labeled mass spectrometry).

| | qNMR | Traditional method |
|---|---|---|
| Sample 1 (%) | 83.06 | 84.59 |
| Sample 2 (%) | 83.51 | 84.67 |
| Sample 3 (%) | 82.71 | 83.39 |
| Sample 4 (%) | 83.16 | 84.74 |
| Sample 5 (%) | 83.51 | 84.21 |
| Sample 6 (%) | 82.92 | 85.75 |
| Sample 7 (%) | 83.26 | 83.61 |
| Mean | 83.16 | 84.42 |
| $S$ (%) | 0.30 | 0.79 |
| CV (%) | 0.36 | 0.93 |
| $F$ | 0.141 | |
| $f_{\alpha}(\nu_1,\nu_2)$ | 4.28 | |
| $\frac{1}{f_{\alpha}(\nu_1,\nu_2)}$ | 0.233 | |
| $f_{\alpha}(\nu_1,\nu_2) < F < \frac{1}{f_{\alpha}(\nu_1,\nu_2)}$ | Not agreed | |
| $t$ | 0.652 | |
| $t_{\alpha,(n_1+n_2-2)}$ | 2.18 | |
| $\lvert t\rvert < t_{\alpha,(n_1+n_{2-2})}$ | Agreed | |

of NMR is proportional to the square of number of scans). In addition, its precision can be enhanced by spending more sample amounts per test. Moreover, the total experiment time of qNMR was about 1 h (including weighing, dissolving and scanning), much less than the traditional method (3 days, including weighing, hydrolysis of 48 h, LC–MS determination). Therefore, this qNMR method is a more precise and promising method for the purity determination of peptide.

## 4. Conclusions

A precise measurement method was presented for the purity of valine with advantages of: (1) all visible peaks in the NMR spectrum were assigned by identification of impurities; (2) no overlap between the two quantitative peaks was confirmed. The intraday CV of this method was 0.052%. The interday CV during 8 months was 0.071%, which is the lowest among the reported references for purity of amino acid. This method greatly enhanced the precision of qNMR for analysis of small organic compounds to a new level.

A precise measurement method was presented for the purity of peptide by: (1) selecting acesulfame potassium as a suitable internal standard; (2) spiking deuterium oxide to suppress exchangeable protons; and (3) using the CH protons in tyrosine residue for quantification. The CV of this method was 0.49%, which was less than the traditional method with the hydrolysis reaction (0.85%). The experiment time of qNMR is about 1 h, much shorter than that of traditional method (3 days).

A new approach was presented to directly determine the purity of peptide, with simpler operation, shorter analytical time and higher precision. By clearing the exchangeable proton peaks with spiking deuterium oxide, the unexchangeable proton peaks among that NMR spectrum area will be more isolated. It is significant for biomolecular analysis because the NMR peaks for large molecule such as peptide are too crowded to select an isolated peak for qNMR. Moreover, the traditional method requires the hydrolysis reaction with complicated operation, which will cost a long analytical time and the CV is larger.

A peptide is a polymer of 20 kinds of common amino acids. Since 19 kinds of common amino acids (except proline) have at least one exchangeable proton ($\delta$=6.5–8.5), and only 4 kinds of amino acids (histidine, phenylalanine, tyrosine and tryptophan) have unexchangeable protons in the same area ($\delta$=6.5–8.5), the unexchangeable protons in that area is not crowded as that in area of methyl and methylene ($\delta$=0.5–4.6) if exchangeable protons were all removed. This approach is suitable for peptide.

This method can be easily applied to any peptide or protein containing these four amino acid residues, because these residues have unexchangeable protons at 6.5–8.0 in the NMR spectrum. For other peptides and proteins, if an isolated proton can be found in their NMR spectra, this method can also be applied.

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