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

Ting Huang [a], Wei Zhang [a], Xinhua Dai [a,*], Xiaoguang Zhang [b], Can Quan [a], Hongmei Li [a], Yi Yang [b]

[a] Division of Chemical Metrology and Analytical Science, National Institute of Metrology, Beijing 100013, China
[b] College of Science, Beijing University of Chemical Technology, Beijing 100029, China

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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

* Corresponding author. Tel.: +86 10 64524787; fax: +86 10 64271638.
E-mail addresses: daixh@nim.ac.cn, xhdai_75@iccas.ac.cn (X. Dai).

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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

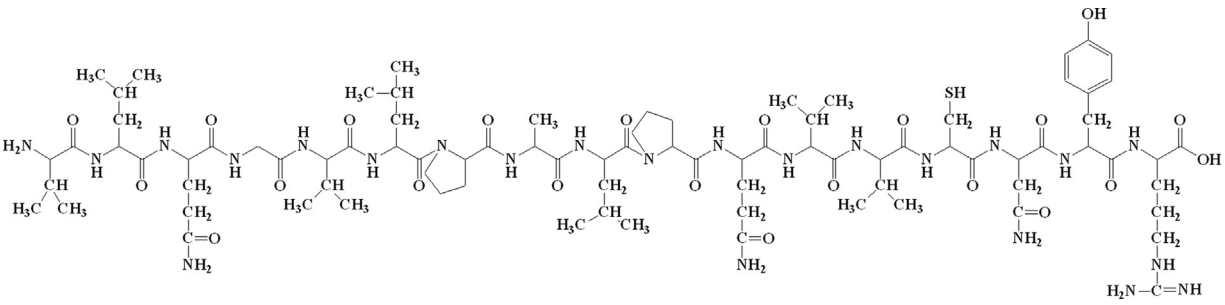

Fig. 1. Structure of peptide T5.