Concentration Measurement of Amino Acid in Aqueous Solution by Quantitative ^1H NMR Spectroscopy with Internal Standard Method

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We describe a procedure to determine concentrations of amino acid standard solutions by quantitative NMR spectroscopy using an internal standard. The measurement samples were prepared by solvent exchange to remove any intense solvent signal in the ^1H NMR spectra. The method was demonstrated on valine aqueous solutions of different concentrations. The accuracy of the measured concentrations that fell well within the range of the expanded uncertainty is also discussed. All of the results are in good agreement with the preparation values. We believe that this approach should be useful to determine the concentrations of standard solutions whose solute components are difficult to weigh because of extremely small amount or hygroscopicity.

Keywords Amino acid, qNMR, standard solution, ^1H NMR, internal standard method, concentration, uncertainty

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Introduction

Nuclear magnetic resonance (NMR) spectroscopy has been widely used for the qualitative analysis of molecules. Recently, quantitative analysis using ^1H NMR spectroscopy has been reported.¹⁻⁴ The quantitative NMR technique uses a compound whose structure differs from the compound to be measured as a reference material. This method was examined as a new approach for rapid purity determination, and was eventually adopted for practical application. It was also used for accurate purity evaluation in reference material production.^{5,6}

In the purity determination by NMR spectroscopy, the measurement accuracy is influenced by the amount of the sample and the resolution of the balance. When only a few milligrams of sample is available, a high-resolution balance, such as an ultra-micro balance, must be used for sample weighing to achieve accurate determinations. In general, not the purity of the material, but the concentration of the standard solution is necessary in almost all kinds of instrumental analyses, including chromatographic techniques. The reason why the material purity should be known is that the concentration of a standard solution is usually dependent on the preparation value of a material of known purity.⁷ If we can determine the concentration of the standard solution directly, it will not be necessary to know the purity. The quantitative NMR technique is a potentially useful analytical method for a concentration determination of a solution. However, as far as we know, there has been no report concerning a direct determination of the solute concentration in a standard solution by a conventional quantitative NMR measurement with an internal standard (IS).

There are several methods for concentrations determination of matrix samples⁸ and low-concentration samples⁹ by quantitative ^1H NMR spectroscopy in a deuterium solvent. However, those methods are not appropriate for a concentration determination of a conventional standard solution. The pre-saturation method with IS,¹⁰ the external standard method, and the electronic reference to access *in vivo* concentrations (ELETIC) method have been used for concentration measurements of standard solutions.^{9,11,12} These methods have been optimized for the measurement of samples prepared with conventional solvents. However, the instability of the baseline near the pre-saturated signal has increased the measurement variation, and limited the signal areas for quantification. In the external standard method and the ELETIC method, the measurement density fluctuation directly affects the accuracy of the measurement results. Moreover, these methods are not suitable as general concentration measurement methods because special instrumentation and correction of some factors are needed.

In this paper, we describe an approach for concentration measurements of standard solutions by ^1H NMR spectroscopy with IS, and discuss the accuracy of this approach. Quantitative NMR spectroscopy with IS is the most accurate approach in quantitative NMR measurements because any NMR equipment instability can be suppressed by measuring the analyte and the standard at the same time. Measurement samples were prepared by solvent exchange to any remove solvent signal in the ^1H NMR spectrum. Standard solutions of several concentrations were measured, and the accuracy was discussed. The uncertainty was evaluated and compared with that of the gravimetric method. We used valine (Val) as the study sample because it possesses such physical properties as non-volatility and water solubility. Finally, this approach was applied to arginine (Arg), a hygroscopic amino acid.

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Experimental

Apparatus and parameters

^1H NMR spectra were obtained with a VNS 600 spectrometer (Agilent Technologies, California, USA) operating at 599.90 MHz with a ^1H resonance frequency. The probe was a Varian ^{15}N - ^{31}P / ^1H - ^{19}F dual-broadband probe equipped with a pulsed field gradient (PFG). The temperature was regulated at 25°C. The typical ^1H NMR experimental parameters were as follows: 59523.8 Hz (99.2 ppm) spectral width, 4.0 s acquisition time, 13.0 μs (90°) pulse width, 60 s relaxation delay, and 32 transients acquired. In this NMR measurement, the spectral width was set to a large range in order to ignore any influence of the bend in the NMR spectrum side, which was effected by a digital filter. The inversion recovery pulse sequence was used for T_1 determinations. Data processing was performed using a MestReNova Ver. 9.0.1. The zero-filling was not use in this data process because the measurement results were not changed whether zero-filling was set or not. Further, other window functions were not used in the data process.

All signals were integrated outside of the ^{13}C satellite signal.¹³

Weighing was performed on a semi-micro balance with a 0.01-mg resolution (XS205, METTLER TOLEDO, Giessen, Germany). For solvent evaporation, a compact evaporator (TGM Co., Ltd., Tokyo, Japan) was used. A TGA instrument (Q5000 SA, TA Instruments, Delaware, USA) was used to evaluate the absorbed moisture in Arg.

Materials

The certified reference materials (CRMs) of Val and Arg with a purity of $(0.998 \pm 0.002)\text{kg kg}^{-1}$ as analyte were obtained from National Metrology Institute of Japan/Advanced Industrial Science and Technology (NMIJ/AIST).¹⁴ The CRM of potassium hydrogen phthalate (PHP) with a purity of $(1.0000 \pm 0.00027)\text{kg kg}^{-1}$ as IS was obtained from NMIJ. Deuterium oxide (D_2O) was purchased from Acros Organics (NJ, USA).

The amino acid aqueous solutions and the IS aqueous standard solution were prepared by the gravimetric method with a semi-micro balance. PHP in a D_2O solution of 5000 mg kg^{-1} was prepared for ^1H NMR measurements. Val in a H_2O standard solution of 5000 mg kg^{-1} was prepared by dissolving Val powder. Then, solutions having concentrations of 125 to 2500 mg kg^{-1} were prepared by one- or two-step dilution of a standard solution of 5000 mg kg^{-1} . An Arg standard solution of 5000 mg kg^{-1} was prepared. Hygroscopic Arg was prepared in the TGA instrument at 80% relative humidity (RH), and the water content was estimated from the change of the mass.

Preparation of a measurement sample

At first, approximately 1 mL of an amino acid aqueous solution was added into a vial and weighed. The solvent of this solution was evaporated with a compact evaporator. Then, 1 mL of an IS standard solution was added to the sample after evaporation with weighing. These masses were weighed by a semi-micro balance. This sample solution was measured by NMR spectroscopy.

Quantification of amino acid standard solution

The concentration was calculated using the following equation:

$$C_x = \frac{S_x}{S_{\text{std}}} \frac{N_{\text{std}}}{N_x} \frac{M_x}{M_{\text{std}}} \frac{m_{\text{std}}}{m_x} C_{\text{std}}, \quad (1)$$

Fig. 1 Procedure for the preparation of a simple column electrode, S-CE.

where C (mg kg^{-1}) is the concentration, S is the integral value of a signal, N is the number of protons generating the signal, M is the relative molar mass and m (g) is the mass of the solution; the x subscript represents the analyte, and the std subscript represents IS. Quantitative analysis was carried out for every signal in the molecule, and concentration was calculated.

Results and Discussion

Concentration determination of a Val standard solution

At first, ^1H NMR measurement of Val in an aqueous solution was carried out. Measurement samples containing Val at different concentrations and IS were prepared and used for ^1H NMR measurements. In the preparation procedure, we were able to weigh the solution with a gram-scale, so that a high-resolution balance, such as an ultra-micro balance, was not needed. Solvent removal suppressed the detection sensitivity reduction by dilution. In addition, the influence of an excess solvent signal on the dynamic range was decreased. For these reasons, the measurement sample for quantitative NMR spectroscopy could be easily prepared at a low cost.

The ^1H NMR spectrum of the measurement sample is shown in Fig. 1. The ^1H NMR spectrum is similar to that obtained by weighing the Val powder and IS. The HOD signal produced by proton exchange between Val and D_2O was observed. The IS signal of PHP was separated sufficiently from the signals of Val. In our previous work, we confirmed whether the measurement sample in which Val and PHP were dissolved in D_2O was stable, and did not react with each other for more than 24 h.¹ The concentration was determined with all of the signals in the ^1H NMR spectrum because the three signals of Val were well separated and the HOD signal and the IS signal were clearly separated from the Val signals (all ^1H NMR spectra are shown in Supplemental data 1). When solvent the removal was insufficient, a less-reliable concentration was obtained because the dynamic range of the signal height and the baseline were influenced by the solvent signal. The intensity of the solvent signal fluctuated in every sample preparation. We have reported that the reliability of a concentration can be evaluated from the agreement of the results from different signals in a molecule in a purity determination.¹⁰ Therefore, concentration was calculated from all of the signals. The results of the quantitative determination of Val standard solutions at concentrations

Table 1 Quantitative results of the Val standard solution

Prepared concentration/ mg kg ⁻¹	Signal	Measurement results				
		Concentration/ mg kg ⁻¹	SD/mg kg ⁻¹ (RSD, %)	Average/ mg kg ⁻¹	Total SD/mg kg ⁻¹ (RSD, %)	The difference with the preparation value, %
124.2	α	125.2	1.7 (1.4)	124.2	1.4 (1.1)	0.0
	β	123.5	1.3 (1.1)			
	γ	124.0	1.0 (0.8)			
255.4	α	257.2	0.9 (0.3)	255.9	1.5 (0.6)	0.2
	β	255.4	2.1 (0.8)			
	γ	255.1	0.5 (0.2)			
513.6	α	518.6	3.0 (0.6)	516.9	2.6 (0.5)	0.6
	β	517.0	3.0 (0.6)			
	γ	515.4	1.0 (0.2)			
1278.5	α	1273.0	6.8 (0.5)	1279.0	7.3 (0.6)	0.0
	β	1282.3	9.1 (0.7)			
	γ	1281.8	1.6 (0.1)			
2559.3	α	2570.3	6.1 (0.2)	2563.8	6.5 (0.3)	0.2
	β	2564.0	1.9 (0.1)			
	γ	2557.2	0.7 (0.1)			
5110.1	α	5120.2	3.0 (0.1)	5098.1	17.8 (0.4)	-0.2
	β	5084.6	7.7 (0.2)			
	γ	5089.3	8.6 (0.2)			

Table 2 Uncertainty budget for Val standard solution depending on the sample concentration

Symbol	Uncertainty factor	Target concentration/mg kg ⁻¹					
		125	250	500	1250	2500	5000
		Relative standard uncertainty, %					
C_x	Repeatability	0.709	0.441	0.318	0.405	0.256	0.379
	Between signal	0.314	0.317	0.148	0.275	0.242	0.371
	Measurement reproducibility	0.636	0.306	0.281	0.298	0.084	0.078
m_s	Mass of sample	0.002	0.002	0.002	0.002	0.002	0.002
m_{IS}	Mass of IS	0.002	0.002	0.002	0.002	0.002	0.002
n_s	¹ H Natural abundance of sample	0.009	0.009	0.009	0.009	0.009	0.009
n_{IS}	¹ H Natural abundance of IS	0.009	0.009	0.009	0.009	0.009	0.009
M_s	Molecular mass of analyte	0.002	0.002	0.002	0.002	0.002	0.002
M_{IS}	Molecular mass of IS	0.001	0.001	0.001	0.001	0.001	0.001
C_{IS}	Concentration of internal standard	0.021	0.021	0.021	0.021	0.021	0.021
$u(C_x)$	Combined standard uncertainty	0.71	0.45	0.32	0.41	0.26	0.38
$U(C_x)$	Expanded uncertainty ($k = 2$)	1.42	0.90	0.64	0.82	0.52	0.76

-ranging from 125 to 5000 mg kg⁻¹ are given in Table 1. All of the results were in agreement with the preparation values within 1.0%, and the measurement reproducibility was approximately 0.5% except for the standard solution of 125 mg kg⁻¹.

The concentration determined from the α -proton signal, which was nearest to the solvent signal, was also in agreement with the preparation value. This indicated that the solvent signal did not influence the measurement results, and reasonable results were also obtained from a signal that was near a high-intensity solvent signal. This result shows that the concentration determined from the α -proton signal was not influenced by a baseline correction, because the HOD signal was sufficiently separated from the α -proton signal. Less than 1% precision could be obtained using the IS method. In our approach, accurate results were easily obtained because the results were not influenced by a non-deuterium solvent as a HOD signal in this study.

The large measurement variation of the 125 mg kg⁻¹ standard solution was caused by a low signal intensity. The measurement

reproducibility of the γ -proton signal of Val was approximately 0.8%. In contrast, the measurement reproducibility of the α -proton and β -proton signals exceeded 1.0%. This difference was explained by the S/N ratio of the quantitative signal. The S/N ratio was 150 for the γ -proton signal and less than 50 for the α -proton and β -proton signals.

Although the measurement reproducibility was influenced by the S/N ratio of the signals, the determined values were in agreement with the preparation value. In addition, although the variation was influenced by the S/N ratio of the signals, there was no bias in this approach. Therefore, we conclude that this approach can directly evaluate the concentration of a standard solution in the case that the component is non-volatile. In general, the signal intensity in the ¹H NMR spectrum is influenced by several factors, including the molecular mass, the number of protons, and the coupling of signals. It is expected that the measurement accuracy would be improved by optimizing the sample concentration and the measurement conditions to

obtain a sufficient signal intensity. An increase of the initial sample size would yield a more intense signal. In a previous report by Saito,¹⁰ the variation reached a plateau when the *S/N* ratio was higher than 1000. Therefore, it is important to set the sample concentration and the number of scans according to the purpose.

Uncertainty estimation of concentration

The uncertainties of the results were estimated in order to evaluate the accuracy of this method. The uncertainty was estimated on the basis of Eq. (1), using an approach similar to a previous purity determination by ¹H NMR spectroscopy.¹ The uncertainties of the precision were estimated by ANOVA. Table 2 gives the uncertainty budget.

The variation between the signals and/or the measurement reproducibility were the main uncertainty factors for all of the samples. The variation between the signals was the main uncertainty factor for 5000 and 2500 mg kg⁻¹ samples. On the other hand, the influence of the measurement reproducibility was larger than that of the variation between the signals for samples of 500 mg kg⁻¹ or less. This could be attributed to the low intensities of the sample signals. The uncertainty factor of the IS concentration was small because the standard solution was prepared with PHP, a high-purity reference material, by the gravimetric method. Moreover, the uncertainty factors of the mass of the sample and that of IS were small because sufficient amounts of the sample and IS were weighed. We also found that the ¹H natural abundance and molecular mass were not influenced to combined uncertainty. Even if we calculate, except for these factors, the difference of the combined uncertainty is under 0.01%. The expanded uncertainty of all samples, except for the 125 mg kg⁻¹ sample was approximately 1%, and no concentration dependence was observed. The major uncertainty factor was the measurement variation in the NMR measurement and the variations were almost the same. NMR measurement reproducibility for the 125 mg kg⁻¹ sample was larger than those for the other samples, as described in "Uncertainty estimation of concentration". An uncertainty of 1% is regarded as being the accuracy that can be used sufficiently in conventional analysis.

The uncertainty of this approach was compared with that of the gravimetric method used in the preparation of standard solutions. When a small amount of sample was prepared by weighing, the resolution of the balance would be the main contributor to the uncertainty. The standard uncertainty of the micro-balance used in this study was 10 µg, based on the calibration certificate. To prepare 1 mL standard solutions whose concentrations would range from 125 to 5000 mg kg⁻¹, we had to weigh 125 µg to 5 mg of the sample. When the uncertainty of the purity was ignored, the standard uncertainties for the concentrations of solutions were calculated as 8.0 to 0.2%. The accuracy of the gravimetric method depends on the amount of sample. The calculated standard uncertainties of less than 2500 mg kg⁻¹ solutions prepared by the gravimetric method were 0.4% or more, which were larger than the uncertainties of our approach. Therefore, our approach is considered to have a potential to decrease the uncertainty of the concentration in the measurement of low-concentration standard solutions.

Application to the determination of hygroscopic amino acid standard solution (Arg)

We applied this method to Arg, a hygroscopic amino acid. The hygroscopicity of Arg was evaluated with the TGA instrument. Arg began to absorb moisture at 40% RH. In this experiment, an Arg standard solution was prepared at 80% RH.

At this humidity, the mass of Arg increased from 10.7650 to 12.9329 mg, which was equivalent to 120.14% of the initial mass (Supplemental data 2). This hygroscopic Arg was dissolved in 2 mL of water, and an Arg standard solution of 6927.5 mg kg⁻¹ was prepared. At this concentration the amount of water contained was not considered.

Then, the concentration of this standard solution was determined by ¹H NMR spectroscopy to be 5355.6 ± 9.2 mg kg⁻¹ (the spectrum shows Supplemental data 3). The value following the plus-minus sign indicates the expanded uncertainty of the determined value. In this analysis, the determined value included the variation between the signals assigned to Arg.

The concentration calculated from the mass before moisture absorption was 5364.9 mg kg⁻¹; the measurement value was in good agreement with this value. From this result, this method can be applied to the direct determination of a standard solution whose component is difficult to weigh for such reasons as hygroscopicity.

Conclusions

The concentration of an amino acid standard solution was determined directly without any bias factor by ¹H NMR spectroscopy using the internal-standard method. We evaluated the accuracy of the quantitative results by comparing them with the preparation value obtained by the gravimetric method. In this approach, all of the quantitative results were in agreement with the preparation values in the range of the expanded uncertainty. In the uncertainty comparison of the standard solution concentration, a small uncertainty by the direct determination approach was noted compared to the uncertainty by the gravimetric method in low-concentration solutions. An accurate concentration measurement for non-volatile components could be achieved by complete solvent exchange. We also studied the hygroscopic amino acid Arg and results similar to Val standard solution were obtained. We conclude that accurate values were obtained in this approach because no bias factor was observed in the determined values. This approach may be useful for standard solutions whose components are difficult to weigh because of rarity or hygroscopicity, and applicable to non-volatile compounds without any solvent dependence.

Supporting Information

This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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