Absolute Quantification of Lipophilic Shellfish Toxins by Quantitative Nuclear Magnetic Resonance Using Removable Internal Reference Substance with SI Traceability

Tsuyoshi KATO,* Maki SAITO,* Mika NAGAE,* Kazuhiro FUJITA,* Masatoshi WATAI,* Tomoji IGARASHI,* Takeshi YASUMOTO,* and Minoru INAGAKI**†

*Japan Food Research Laboratories, Tama Laboratory, 6-11-10 Nagayama, Tama, Tokyo 206-0025, Japan
**Department of Life Science, Faculty of Bioresources, Mie University, 1577 Kurima-Machiya, Tsu, Mie 514-8507, Japan

Introduction

A lipophilic shellfish toxin, okadaic acid (OA), is a large and complex marine toxin produced by dinoflagellates e.g. Dinophysis fortii and Prorocentrum lima. This has been detected in filter-feeding bivalve shellfish, such as oysters, mussels, scallops and clams, which prey on the dinoflagellates having toxin. The conventional method for monitoring toxic shellfish is the mouse bioassay; however, there has been a shift to using instrumental analysis techniques, such as LC/MS, to identify and quantify the target shellfish toxin among various toxins. Consequently, the demand for authentic shellfish toxin standards has risen; yet, the sources of such shellfish toxin standards are limited to toxic shellfish and cultured dinoflagellates. In addition, laborious steps are required for extraction and purification; therefore, purified shellfish toxins are extremely precious.

As a worldwide toxin reference supplier, Canada employed quantitative nuclear magnetic resonance (qNMR) earlier than others. qNMR is one of the best quantitative methods used to calibrate precious materials, including shellfish toxins. It has the following two main advantages: (1) samples can be recovered because qNMR is a non-destructive method, and (2) the NMR signal intensity is theoretically directly proportional to the molar concentration of a sample, so a single measurement is sufficient for accurate quantification without the use of a calibration curve. Furthermore, qNMR has been established as a purity calculation method. The qNMR method which is called “accurate quantitative NMR with internal reference substance (AQARI)” and its equivalent method have achieved reliable traceability to the SI units using certified reference materials (CRMs) as internal standards; they are more accurate than external standard analysis techniques. However, the analysis of large molecules (>800 Da), such as OA and dinophysistoxin-1 (DTX1), is challenging for qNMR, because their complex structural features—numerous proton nuclei and diverse conformations—cause partial spectral overlap. Moreover, the use of CRMs of involatile materials is disadvantageous, because they can cause contamination.

In the present study, OA and DTX1 were quantified based on the AQARI technique. Pyridine and the residual proton in methanol-d4 were used as removable internal standards to limit any contamination. They were calibrated based on a maleic acid certified reference material. Thus, the concentration of OA was traceable to the SI units through accurate quantitative NMR with an internal reference substance. Signals from the protons on the oxygenated and unsaturated carbons of OA were used for quantification. A reasonable accuracy was obtained by integrating between the lower and upper 13C satellite signal range when more than 4 mg of OA was used. The best-determined purity was 97.4% (0.16% RSD) when 20 mg of OA was used. Dinophysistoxin-1, a methylated analog of OA having an almost identical spectrum, was also quantified by using the same methodology.

Keywords Lipophilic shellfish toxin, okadaic acid, dinophysistoxin-1, polyethers, qNMR, AQARI

(Received December 9, 2015; Accepted March 4, 2016; Published July 10, 2016)
were extracted from poisonous shellfish, and supplied by Yasumoto et al. These lipophilic shellfish toxins exhibited almost only one peak on the total ion chromatogram of LC/MS. OA and DTX1 were dried in vacuo in a desiccator containing diphosphorus pentoxide for at least 20 days. NMR test tubes with a 5-mm outer diameter were purchased from Kusano Science Co. (Tokyo, Japan). Methanol-d₄ (99.96% deuterium content) was obtained from Merck KGaA (Darmstadt, Germany), pyridine (guaranteed reagent, 99.8%) from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA), and the maleic acid certified reference material (purity 99.7%, expanded uncertainty was 0.9%, k = 2) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Apparatus
The experimental set up was composed of a semi-micro balance (AG285, Mettler-Toledo, Greifensee, Switzerland), an ultra-micro balance (MSE2.7S, Sartorius AG, Göttingen, Germany), and an NMR spectrometer equipped with a Varian 5-mm indirect probe (Varian NMR System 500, Varian Technologies, Palo Alto, CA, USA).

Preparation of test solutions containing pyridine as an internal standard
Twenty microliters of pyridine was diluted using 4 mL of methanol-d₄. OA (20.1 mg) and the maleic acid CRM (2.26 mg) were accurately weighed. Each was then dissolved in 1 mL of the above-mentioned solution to afford test solutions of maleic acid CRM containing pyridine as the internal standard and the methanol-d₄ solution of pyridine containing the maleic acid CRM, respectively.

Preparation of test solutions containing residual proton of methanol-d₄ as an internal standard
The maleic acid CRM (1.46 - 2.64 mg) and ampules of methanol-d₄ (0.669 g (average), ca. 0.8 mL) were accurately weighed and mixed to afford test solutions of maleic acid CRM (N = 5) containing the residual proton of methanol-d₄ as the internal standard. OA (8.99 mg) and DTX1 (4.27 mg) were also accurately weighed and dissolved by the contents in the weighed ampule of methanol-d₄ (0.674 and 0.667 g, ca. 0.8 mL) to prepare the OA (11 mg/mL) and DTX1 (5.1 mg/mL) solutions containing the residual proton of methanol-d₄ as the internal standard.

1H-NMR measurements
For 1H-NMR experiments, the relaxation delay was six-times or above the longest relaxation time (T₁) of pyridine signals to recover over 99% of z-magnetization; T₁ was previously determined by an inversion-recovery test. The following settings were used for AQARI experiments: irradiation frequency, 499.868 MHz; acquisition time, 4 s; relaxation delay, 60 s; probe temperature, 7 or 25°C; spectral width, 40.332 ppm; FID data points, 161288; number of scans, 8 - 2560; spinning, off; dummy scans, two times; 1H decoupling, MPOF; pulse angle, 90°; pulse width, 10.4 μs.

Data processing
Data were processed with VnmrJ software Ver. 2.3 supplied by the manufacturer (Varian Inc.). Fourier transformations were performed on 262144 data points. All proton chemical shifts were referenced to the residual proton signal in methanol-d₄ (CD-HOD) at 3.300 ppm. The phase of all spectra was collected manually while observing the spectral line shape. The baseline of the spectra were adjusted horizontality, and each signal area ratio was calculated by software functions. An integration range was individually optimized based on the line width of the signals and the space between the signals. The spectra were integrated using the spectral bucketing technique with 0.002-ppm-sized buckets. The area ratio of each signal was calculated by adding all buckets larger than buckets arising from the baseline (noise level).

Determination of the quantity of pyridine as internal standard
The quantity of pyridine in the solution was determined using the 1H-NMR spectrum of the maleic acid-pyridine solution by the following Eq. (1):

\[ \text{Value}_A = \frac{I_A}{I_{IS}} \times \frac{N_{IS}}{N_A} \times \frac{M_A}{M_{IS}} \times W_{IS} \times P_{IS}, \]

(1)

\[ P_A = \frac{I_A}{I_{IS}} \times \frac{N_{IS}}{N_A} \times \frac{M_A}{M_{IS}} \times W_{IS} \times P_{IS}, \]

(2)

where Valueₐ is the quantity of a target compound (g); Iₐ is the area ratio of each individual signal resulting from the target compound; Iₐ is the area ratio of the internal standard; Nₐ is the number of protons of the target compound; Mₐ is the mole weight of the target compound; Mₐ is the mole weight of the internal standard; Wₐ is the weight (g) of the internal standard; Pₐ is the purity (w/w%) of the internal standard; Pₐ is the purity (w/w%) of the target compound; and Wₐ is the weight (g) of the target compound. The quantity was calculated using the total area of pyridine signals (P1 – P3 are shown in Fig. 1) as 5 protons to be 0.00591 g.

Determination of the purity of CD2HOD in methanol-d₄
The quantity of CD2HOD was calculated using the 1H-NMR spectra of a solution of maleic acid CRM dissolved in methanol-d₄. Employing maleic acid as the internal standard, the CD2HOD purity was determined to be 0.117% (0.1% RSD; N = 5) using Eq. (2) provided above.

Evaluation of the dispersion in the quantitative value affected by the phase corrections
The 1H-NMR spectrum of DTX1 was measured by 1, 8, and 16384 transient accumulations. The signal area ratios were obtained by 20 repetitions of integration and calibration; the deviations were also used for repeatability calculations.

Concentration dependence of the OA spectra
1H-NMR spectra of OA were obtained for 0.01, 0.09, 0.5, 1, 4, and 11 mg/mL of OA. Accurately weighed OA samples (ca. 1, 4, and 9 mg) and methanol-d₄ (0.61 - 0.68 g, ca. 0.8 – 0.9 mL) were mixed to afford test solutions. OA test solutions containing 0.01, 0.09, and 0.5 mg/mL of OA were prepared by diluting a solution containing 1 mg/mL of OA with methanol-d₄.

Evaluation of trueness
OA quantification was performed in triplicate (from sample solution preparation to qNMR measurement) for all OA concentrations (0.01, 0.09, 0.5, 1, and 4 mg/mL), except 11 mg/mL (single measurement). The trueness (%) was determined by dividing of the individual quantity (%) by the purity of OA.

Results and Discussion
Measurement of OA and DTX1 spectra
1H-NMR spectra of OA and DTX1 are shown in Fig. 1.
The assignment of each signal was based on the reference. Both spectra were very similar because the structural difference between OA and DTX1 is limited to the presence or absence of the methyl group at position 35. The proton signals belonging to the saturated hydrocarbon groups were inappropriate for quantification because of the degree of overlap. In contrast, signals A – K, arising from the protons on unsaturated or oxygenated carbons, were moderately- or well-separated and, hence, they were used for quantity calculations. Signals F, H and J were composed of more than two protons. Also signal C composed of two insufficiently separated signals were thus treated together. However, signal D, near to the water signal, was excluded from quantification. The measurement temperature was set so that the water signal position was adjusted to the center of signals C and E.

**Determination of OA purity**

A spectrum having sufficient signal intensity (S/N >1000) was obtained by eight transients using 20.1 mg/mL of OA. The area between the lower and upper 13C satellite signals has been reported to be a reliable integration range to obtain accurate area ratios; however, this area was only applicable to signals E, FG and P1 - P3. Signals A - K and P1 - P3 were integrated by a spectral bucketing and binning technique with 0.002-ppm-sized buckets. The signal area ratios were calculated as a sum of bins, which were larger in size than that of the baseline region (Range A, Fig. 2). The average quantified value based on the integration of Range A using signals A - K and total of P1 - P3 as the internal standard was 97.6% with RSD 0.89% (Table 1) using the Eq. (2) provided above. In addition, the signal area ratios for E and FG were also integrated from the area between the 13C satellite signals (Range B). The quantified values of signals E and FG employing Range B were 97.3% (0.20% RSD) and 97.4% (0.02% RSD), respectively. Those are very well consistent with that by Range A. Hence, the purity of OA was established to be 97.4% with traceability to the SI units.

**Applicability of residual proton in methanol-d₄ as an internal standard**

The residual proton in methanol-d₄, CD₂HOD, was also suited to be the internal standard. Although T₁ of the residual proton (10 s) was slightly longer than that of pyridine (P1, 7.0 s, P2, 7.5 s, and P3, 6.9 s) in methanol-d₄, the concentration of the residual proton was consistently stable (within an error of 0.1% RSD) for as much as 10 h. However, the signal of CD₂HOD was proximally close to signals J and K, so the measurement temperature was optimized so as to avoid any overlap. The area ratio of CD₂HOD was successfully determined to be 99.6%.
when compared to the theoretical value using solely CD$_2$HOD without OA at 7°C. Table 2 gives the quantified results of OA (11 mg/mL) and DTX1 (5.1 mg/mL) using CD$_2$HOD as the internal standard. Those values of OA (Table 2) were consistent with those from when pyridine served as the internal standard (Range A, Table 1). DTX1 was quantified by the same method.

The amount of CD$_2$HOD in the ampules of methanol-$d_4$ was found to be consistent within the same lot of the reagent (0.1% RSD, $N = 5$). In order to use the pyridine internal standard solution, it had to be prepared before every commencing use. Thus, the use of the residual proton CD$_2$HOD improved the working efficiency by reducing the sample preparation steps and eliminating any external calibration. It succeeded in obtaining traceability to the SI units for absolute quantity measurements of shellfish toxin standards.

### Table 1 Calculated purity of OA (20.1 mg/mL) using pyridine as the internal standard (IS)

| Signals  | Number of protons | Integration Range | Width, ppm | Purity Value, % | % RSD |
|----------|-------------------|-------------------|------------|-----------------|------|
| P$_1$ as IS | 2 A | 0.300 | — | — |
| P$_2$ as IS | 1 A | 0.260 | — | — |
| P$_3$ as IS | 2 A | 0.280 | — | — |
| A | 1 A | 0.232 | 96.4 | 0.2 |
| B | 1 A | 0.200 | 98.7 | 0.1 |
| C | 2 A | 0.230 | 98.5 | 0.1 |
| E | 1 A | 0.206 | 97.3 | 0.2 |
| F | 3 A | 0.182 | 97.0 | 0.1 |
| G | 1 A | 0.154 | 99.0 | 0.3 |
| H | 3 A | 0.208 | 97.0 | 0.1 |
| I | 1 A | 0.106 | 97.8 | 0.2 |
| J | 2 A | 0.132 | 97.4 | 0.1 |
| K | 1 A | 0.140 | 96.9 | 0.3 |
| P$_1$ as IS | 2 B | 0.399 | — | — |
| P$_2$ as IS | 1 B | 0.326 | — | — |
| P$_3$ as IS | 2 B | 0.409 | — | — |
| E | 1 B | 0.372 | 97.3 | 0.2 |
| FG | 4 B | 0.399 | 97.4 | 0.02 |

### Table 2 Calculated purity of OA (11 mg/mL) and DTX1 (5.1 mg/mL) using CD$_2$HOD in methanol-$d_4$ as the internal standard (IS)

| Signals  | Number of protons | Integration Range | Width, ppm | Purity Value, % | % RSD |
|----------|-------------------|-------------------|------------|-----------------|------|
| CD$_2$HOD as IS | 1 A | 0.054 | — | — |
| E | 1 A | 0.206 | 97.8 | 0.07 |
| FG | 4 A | 0.336 | 97.6 | 0.06 |
| CD$_2$HOD as ISDTX1 | 1 A | 0.061 | — | — |
| FG$_{DTX1}$ | 4 A | 0.318 | 97.9 | 0.07 |

however, the magnitude of improvement was significantly different for each signal. Signals A - D had relatively larger phase fluctuations than others because of interference from the intense water signal. The repeatability of signals F, H, and J, which involved multiple protons, was lower than those involving single protons (signals A, B, D, E, G, I, and K), and were superior for quantification because they were less affected by any phase fluctuation.

### Effect of the OA concentration on the signal shape

The effect of the OA concentration on the signal shapes was investigated in cases of spectra using 0.01, 0.09, 0.5, 1, 4, and 11 mg/mL of OA (Fig. 4). Signals A, E, and H, significantly broadened in line shape with reducing the OA concentration. Particularly, signal A essentially disappeared at 0.01 mg/mL of OA. The carboxyl group at position 1 of OA has been reported to form a cavity-like structure with the hydroxyl group at position 24 by intramolecular hydrogen bonding. Therefore, the signal was most likely affected under low concentrations by the significant conformational disorder at the highly flexible part of the OA skeleton around the corresponding protons. Signal A showed a broader line width and a lower intensity than signal B, which is on the opposite side of the double bond. Thus, signal A was unreliable for quantification. For an OA of less than 0.4 mg/mL, signal E was also not reliable, presumably based on the above-mentioned conformational disorder.

### Evaluation of trueness

Figure 5 shows the relationship between the OA concentration (mg/mL) and trueness (%) of quantitative values. The trueness, calculated from signal A and E, tended to deviate from the theoretical value as the concentration decreased. The trueness values calculated from 11 or 4 mg/mL of OA showed nearly 100% of trueness using Range A as well as Range B area.
integration methods; however, the values deviated from the theoretical value for the Range B area integration for low concentrations (>0.1 mg) in comparison to that of Range A. This was an effect of Range B being more sensitive to an increased noise level. The area at the part of the baseline tended to be increased with rising noise level, with the exception of signals A and E, which broadened under less OA concentration (Fig. 4). Therefore, the present study revealed that Range A, where the integration range was optimized for the individual signals, should be employed unless the spectrum is obtained from more than 4 mg/mL of OA. Considering the subjectivity caused by a phase correction, signal F or FG was the best choice for OA quantification. Although accurate quantification of OA was accomplished by qNMR, the accuracy was admittedly dependent on the amount of OA available. For qNMR using the aforementioned signals, the trueness was 100.1% (0.39% RSD) for 4 mg/mL, 100.1% (1.3% RSD) for 0.09 mg/mL, and 112.1% (1.5% RSD) for 0.01 mg/mL. We thus judged that the authenticity of the purity of OA and DTX1 should be obtained from the data using more than 4 mg of toxins.

Conclusions

OA (20.1 and 8.99 mg) and DTX1 (4.27 mg) were weighed, used for preparing of test solutions, and were measured utilizing an NMR instrument with a proton resonance frequency of 499.87 MHz. OA and DTX1 were accurately quantified by qNMR, using the well-separated proton signals attached to the oxygenated carbons, to afford reference toxin standards having authentic purity (97.4 and 97.9% respectively). The residual proton CD2HOD in methanol-d4 was beneficially used as an internal standard, which could be removed afterward, and to avoid contaminating the precious shellfish toxins. Using the residual proton significantly improved the working efficiency due to a simplification of the sample preparation and calibration. The applicability of OA quantification by AQARI was established using the calibrated OA standard. Our findings showed that the integration range should be optimized for individual signals, unless a sufficient amount of OA affords a spectrum having adequate intensities; more than 0.1 mg/mL of OA is highly recommended to use for AQARI in order to suppress any deviation (less than 5%) from the true value.

References

1. T. Yasumoto, Y. Oshima, and M. Yamaguchi, Bull. Jpn. Soc. Sci. Fish., 1978, 44, 1249.
2. K. Tachibana, P. J. Scheuer, Y. Tsukitani, H. Kikuchi, D. V. Engen, J. Clardy, Y. Gopichand, and F. J. Schmitz, J. Am. Chem. Soc., 1981, 103, 2469.
Fig. 5 Correlation between the OA concentration and its trueness, calculated from signals A, B, C, E, F, G, and FG. Each data point represents three individual experiments, except for 11 mg/mL (single measurement). (A), signals A, B, and C (Range A); (B), signal E (Ranges A and B); (C), signals F, G, FG (Range A), and signal FG (Range B).

3. T. Yasumoto, Y. Oshima, W. Sugawara, Y. Fukuyo, H. Oguri, T. Igarashi, and N. Fujita, Bull. Jpn. Soc. Sci. Fish., 1980, 46, 1405.
4. Y. Murakami, Y. Oshima, and T. Yasumoto, Bull. Jpn. Soc. Sci. Fish., 1982, 48, 69.
5. J. Alexander, G. A. Auðunsson, D. Benford, A. Cockburn, J. Cravedi, E. Dogliotti, A. D. Domenico, M. L. Fernández-Cruz, J. Fink-Gremmels, P. Fürst, C. Galli, P. Grandjean, J. Gzyl, G. Heinemeyer, N. Johansson, A. Mutti, J. Schlatter, R. V. Leeuwen, C. V. Peteghem, and P. Verger, EFSA J., 2008, 589, 1.
6. T. Suzuki and M. A. Quilliam, Anal. Sci., 2011, 27, 571.
7. O. B. Stabell, V. Hormazabal, I. Steffenak, and K. Pedersen, Toxicon, 1991, 29, 21.
8. R. Draisci, L. Croci, L. Giannetti, L. Cozzi, L. Lucentini, D. D. Medici, and A. Stacchini, Toxicon, 1994, 32, 1379.
9. M. A. Quilliam, J. AOAC Int., 1995, 78, 555.
10. I. W. Burton, M. A. Quilliam, and J. A. Walter, Anal. Chem., 2005, 77, 3123.