Journal of the Korean Magnetic Resonance Society **2020**, *24*, 30-37
DOI 10.6564/JKMRS.2020.24.1.030

Purity assessment using quantitative NMR: establishment of SI traceability in organic analysis

Kihwan Choi*

Division of Chemical and Medical Metrology, Korea Research Institute of Standards and Science, Yuseong, Daejeon 34113, Republic of Korea

Received Mar 17, 2020; Accepted Mar 19, 2020

Abstract Quantitative nuclear magnetic resonance (qNMR) has been gaining attention as a purity assessment method. In particular, qNMR is recognized as the primary method to realize the Internal System of Units (SI) in organic analysis. The capability of quantitative analysis is recognized as the beginning of NMR development. NMR signals are proportional to the number of nuclei and qNMR has been used in various fields, such as metabolomics and food and pharmaceutical analysis. However, careful sample preparation and thorough optimization of measurement parameters are required to obtain accurate and reliable results. In this review, quantitative methods used in qNMR are discussed, and the important factors to be considered also introduced. The recent development of qNMR techniques including combination with chromatography and, multidimensional NMR are also presented.

Keywords Purity assessment, qNMR, SI traceability, internal standard

Introduction

Metrological traceability for chemical measurement is important for ensuring the reliability of measurement results. Metrological traceability is realized by an unbroken calibration hierarchy and link to the Internal System of Units (SI).[1] The SI traceability in organic analysis is realized through primary calibrators. The certified values of primary calibrators are linked to the SI using a purity assessment methodology.[2] The mass balance method has been generally used for the purity assay of the calibrators.[3] Various analytical techniques including gas chromatography, liquid chromatography, Karl Fischer titration, and thermal gravimetric analysis have been employed, and the amount of impurities detected in these techniques were subtracted to obtain the purity values of the calibrators. However, the mass balance method cannot be applied to organic substances that are involatile and lack an inherent chromophore. If all the impurities are not properly detected and quantified, the purity of the calibrator can be overestimated. Additionally, the mass balance method requires considerable effort and resources.

Recently, quantitative nuclear magnetic resonance (qNMR) has been used for purity assessment of calibrators[4] in addition to pharmaceutical, natural products and food analysis.[5-8] The peak area of the NMR signal which is proportional to the number of nuclei present, enables the measurement of the amount of analyte in solution. Thus, the purity assessment procedure can be simplified using qNMR, which can be employed for wide range of organic substances as a general method. The metrological traceability of qNMR to the SI was discussed.[9] In addition, the unique NMR spectrum of each

* Correspondence to: **Kihwan Choi**, Division of Chemical and Medical Metrology, Korea Research Institute of Standards and Science, Yuseong, Daejeon 34113, Republic of Korea, Tel: +82-42-868-5557; E-mail: kihwan.choi@kriss.re.kr

substance enables the identification of substances in solution. The Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) Woking Group on Organic Analysis (OAWG) is actively investigating the potential of qNMR,[10-12] and national metrology institutes are incorporating qNMR to develop certified reference materials.[13]

Basics of Purity Assessment and Uncertainty Evaluation

Purity is determined by internal or external standard methods. Both methods rely on the dependency of NMR intensity on the number of nuclei.

Internal standard method – In the internal standard (IS) method, known amount of standard is added to a solution containing the anlayte. As NMR signals of the analyte and IS are acquired in a single measurement, such signals are relatively less sensitive to experimental conditions. The proper selection of IS is critical to obtaining accurate results. IS should have at least one resonance that does not overlap with that of analyte. Moreover, IS should not react with the analyte. Additionally, IS should be soluble in the same solvent as the analyte. High purity and simple chemical structure are additional criteria of for ideal IS. In the IS method, the purity of the analyte can be calculated using the following equation.

$$P_{\mathrm{a}} = \frac{I_{\mathrm{a}} N_{\mathrm{s}} M_{\mathrm{a}} W_{\mathrm{s}}}{I_{\mathrm{s}} N_{\mathrm{a}} M_{\mathrm{s}} W_{\mathrm{a}}} P_{\mathrm{s}} \qquad (1)$$

where P is purity as the mass fraction, I is the integrated signal area, N is the number of ^{1}H atoms contributing to the signal area, M is the molecular weight, W is the weight, and the subscript ‘a’ and ‘s’ represent the analyte and the internal standard, respectively.

The combined standard uncertainty is calculated using Equation 2.

$$u_c(P_{\mathrm{a}}) = \sqrt{\left(\frac{u(I_{\mathrm{a}}/I_{\mathrm{a}})}{I_{\mathrm{a}}/I_{\mathrm{a}}}\right)^2 + \left(\frac{u(M_{\mathrm{a}})}{M_{\mathrm{a}}}\right)^2 + \left(\frac{u(M_{\mathrm{s}})}{M_{\mathrm{s}}}\right)^2 + \left(\frac{u(W_{\mathrm{a}})}{W_{\mathrm{a}}}\right)^2 + \left(\frac{u(W_{\mathrm{s}})}{W_{\mathrm{s}}}\right)^2 + \left(\frac{u(P_{\mathrm{s}})}{P_{\mathrm{s}}}\right)^2} \qquad (2)$$

Currently, there are many endeavors to develop a wide variety of IS, as the chemical structure and properties of analytes are diverse.[14-18] It has been reported that the precision of the IS method is higher than that of the external standard method.[19] However, potential peak overlap between the analyte and IS is a major drawback.

External standard method – In the external standard method, the standard is not added to solution containing the anlayte. Therefore, peak overlap between the analyte and IS can be avoided. A few variations of this method have been used. One such method utilizes a coaxial insert containing a standard solution in an NMR tube containing analyte solution.[20] Peaks of the analyte and standard appear in the NMR spectrum, using the known amount of standard in the coaxial tube, the purity of the analyte can be calculated as in the IS method. The procedure is simple; however, signal overlap can still occur just as in the internal standard method. A more general method is to acquire the NMR spectra of the standard and analyte solutions separately.[21] After the response of the NMR instrument is calibrated with a standard solution, the NMR spectra of the analyte solutions are obtained with the same experimental parameters. Absolute peak areas of the standard and analyte are calculated, and the following equation is used to obtain analyte purity.

Instead of the using a standard solution, a third method uses electronically generated signals as a reference in quantitative analysis. Several approaches including Electronic REference To access *In vivo* Concentrations (ERETIC),[22-24] Amplitude-corrected Referencing Through Signal Injection (ARTSI)[25] and, QUANTification by Artificial Signal (QUANTAS)[26] have been developed. These approaches have not been applied for purity analysis yet; however, they have great potential for such applications. In ERETIC, the electrical reference signal generated by an

additional electronic device is transmitted through the carbon coil. The ERETIC signal is mixed with the NMR signals of the analytes. Then, the signals are treated and the ERETIC signal is shown as a regular NMR peak in the spectrum. The ERETIC signal calibrated with the reference solution can be used for the quantitative analysis of multiple sample solutions. ARTSI improves the accuracy of ERETIC, especially when the composition of the sample solution, such as the solvent or salt concentration, is different from that of the reference solution. In ARTSI, the electronically generated signal is not sent through the NMR probe as in ERETIC; instead, a directional coupler is used to introduce the signal through the full receive path of the spectrometer. When the receptivity of the probe coil is changed, the electronically generated signal is also changed accordingly. QUANTAS is the approach using an artificial signal added through software. In this approach, the digital signal is scaled to compensate for the intensity change of the analyte solution signal as the experimental parameters are changed.

Experimental Parameters for Accurate Measurement

To obtain reliable and, accurate measurement results, the experimental parameters were carefully considered.

Sample preparation – Since purity value is directly proportional to mass, accurate and precise weighing of the analyte and IS is essential in qNMR analysis. Saito et al. showed that the uncertainty contribution from weighing was the largest among other factors in assay results.[27] The high-resolution balance, larger amount of sample, and light tare weight were beneficial in improving accuracy and minimizing uncertainty.The properties of the solvent affect the chemical shift in NMR spectroscopy. The interaction between analyte and solvent can shift 4.6 ppm in ^{1}H NMR.[28] Thus peak overlap between analyte and IS can be overcome by changing solvent. And broad water peak could be sharpened to avoid overlap.[29]

Data acquisition – NMR optimization is important in obtaining accurate purity assay results. Relaxation delay is one of the most important parameters. If the relaxation delay is not sufficient for the *z*-magnetization recovery, the peak area becomes smaller and a biased result is obtained.[30] Generally, it is advised that the relaxation delay should be at least 5 times larger than the longest spin-lattice relaxation time. The signal-to-noise ratio (S/N) also affect accuracy and uncertainty.[31] When S/N<30, the purity values were different from the median value, which was calculated using 1359 NMR spectra having various S/N values ranging from 3 to 72000. Additionally, the uncertainty decreased from 3% to 1% as the S/N increased from 30 to 100. As such, the S/N can be improved by acquiring more scans because S/N is proportional to the square root of the number of scans.

Hyphenation with Chromatography

Separation of impurities by chromatography – The overlap between the resonances of the analyte and impurities is a concern in qNMR.[32] Peaks of structurally similar impurities are prone to overlapping with those of the analyte, and this leads to the overestimation of analyte purity. The separation capability of chromatography has been utilized to circumvent such an overlap. In the offline coupling approach, IS was added to the sample solution. Then the analyte and IS were separated from the impurities and collected using liquid chromatography.[33] Since the eluent contained a solvent with a large number of protons, solvent exchange with a deuterated solvent was necessary. In other ways, solvent suppression during NMR measurement was required. Zhang et al. developed an internal standard recovery correction (ISRC) method to correct for the incomplete collection of eluents.[34] To compensate for the loss of compounds during LC and the sample preparation procedure, the correction factor was calculated by comparing the LC analysis results before and after fraction collection. When ISRC was applied to the purity assay of avermectin B_{1a}, purity values of 7 independent sample were

92.81-93.29%. These values were corrected from 77.08-171.31 % which were determined by qNMR.
Continuous-flow LC-NMR was used for qualitative analysis.[35] When gradient elution was used for LC separation, IS were added to the mobile phase because the NMR spectrum acquisition condition was not consistent. For isocratic elution, a solution containing IS was injected into the separation column during the chromatographic run of the sample solution. Since the chromatographic peak volume was larger than the NMR cell volume of the instrument, NMR chromatograms were binned to increase the signal-to-noise ratio. Saito et al. analyzed IS and analyte solutions injected consecutively onto the separation column.[36] By comparing the peak areas of IS and analyte, the purity of the analyte was determined. Multiple and structurally similar analytes can be quantified using continuous-flow LC-NMR. However, expensive deuterated solvents are needed for the preparation of LC mobile phases. Furthermore, sophisticated optimization of LC and NMR measurements is required. Qualitative information from the NMR spectrum can be used in LC. Quantitative analysis of co-eluting α- and γ-linolenic acid could be achieved with LC-NMR, because the chemical shift of the methyl group of the two analytes were different in the ^{1}H NMR spectrum.[37] Moreover, linoleic acid, which overlapped with γ-linolenic acid in the ^{1}H NMR spectrum, was separated using LC before quantification.

Combination with chromatography – Structurally related impurities have been analyzed by chromatography with flame ionization detector (FID), absorbance detector, or charged aerosol detector (CAD). However, these detection techniques have drawbacks. FID connected with GC can be used only for volatile analyte. The response of CAD is dependent on the mobile phase composition.[38] To obtain the exact mass of impurities with absorbance detection, the absorptivity coefficients of each impurity are needed. The response factor (RF) of chromatographic detection was determined using NMR spectroscopy to correct bias.[39] IS was added to the sample solution and the molar ratio of the anlayte and IS was calculated by comparing the corresponding peak areas from NMR spectrum. RFs of herbicides which were determined by LC coupled with absorbance detection and by qNMR were compared to validate this approach. Kitamaki et al. obtained relative molar sensitivity for analytes using the molar ratio determined from qNMR and the response ratio estimated from chromatography as shown in Figure 1.[40] The concentrations of naphthalene and benzo[*a*]pyrene in NIST SRM 1647f measured using qNMR/GC and qNMR/LC were in agreement with the certified values of each analyte. When signal overlap occurred between the analyte (2-chlorophenol) and impurity (phenol) in the ^{1}H NMR spectrum, the purity of 2-chlorophenol was estimated by qNMR/GC using RMS. This value was confirmed by measuring the phenol content through GC-FID. In addition, the purity of 4-chlorophenol was determined by qNMR/GC and qNMR/LC.

In the peptide impurity corrected qNMR (PICqNMR) approach, the purity of target peptide assessed by qNMR was corrected by subtracting the amount of related pepetide impurities measured by LC-high resolution mass spectrometry. The purity value of angiotensin I from PICqNMR was in agreement with those from the mass balance method, peptide impurity corrected amino acid analysis, and peptide impurity corrected elemental analysis.[41] This approach was used to develop the CRM for angiotensin II.[42]

Multidimensional NMR

Quantitative heteronuclear single quantum correlation (qHSQC) – Peak overlap in one-dimensional NMR spectrum can be diminished by adding a second dimension.[43] Two-dimensional NMR techniques are useful in obtaining structural information of compounds in complicated mixtures. However, the application of 2D NMR techniques to quantitative analysis is not straightforward because

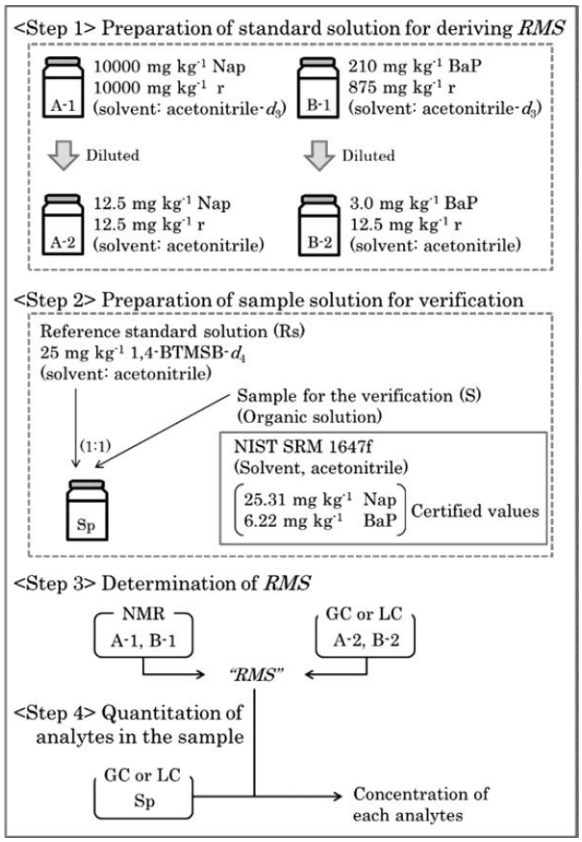

Figure 1. Scheme of the combination of chromatography and qNMR. Adapted with permission from Ref. 40.

signal attenuation occurrs during polarization transfer.

The peak volume is maximized when the polarization transfer time (Δ) is set as $\Delta = 1/2^1J_{CH}$. Heikkinen et al. optimized Δ-values instead of using an average $^1J_{CH}$-coupling in HSQC experiments.[44] Multiple HSQC spectra obtained with selected Δ-values were averaged. Furthermore, the Carr–Purcell–Meiboom–Gill (CPMG)-Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) polarization transfer period was used to avoid peak distortion induced by J_{HH} coupling and this technique; this technique was called Q-CAHSQC. However, the duration of the experiments was extended to acquire averaged results. Quick, quantitative HSQC (QQ-HSQC) was developed to address such a drawback.[45] NMR signals corresponding to different INEPT delays were obtained from different sections in a single scan. QQ-HSQC was applied for the analysis of strychnine. Peak integrals acquired from QQ-HSQC were closely matched to those from Q-HSQC or Q-CAHSQC. However, the accuracy of QQ-HSQC was limited due to the finite bandwidth of the heteronuclear pulses and, homonuclear coupling. Koskela et al. further improved the technique by using adiabatic and phase-modulated pulses to increase the accuracy of quantitative analysis using NMR.[46] This quantitative, offset-compensated, CPMG-adjusted HSQC (Q-OCCAHSQC) experiment reduced the attenuation effect of peaks with a larger ^{13}C offset.

The signal attenuation during polarization transfer was compensated by acquiring HSQC spectra with varying repetition times, this technique was named $HSQC_0$.[47] The signal attenuation factor for each peak was calculated by extrapolation and time-zero peak volume could be determined assuming that the signal attenuation had a linear relation with the number of repetitions. However, higher noise prohibited the application of the $HSQC_0$ technique to solution with high concentration of analytes. The gradient-selective HSQC spectra and fast maximum likelihood reconstruction (FMLR) approach were employed for the quantitative analysis of complicated metabolites as shown in Figure 2.[48] Fardus-Reid et al. validated qHSQC techniques by comparing qHSQC results with gravimetric values.[49] The linearity, repeatability, and precision were evaluated. They also investigated the bias and uncertainty contributions from major parameters. The combined uncertainty of homonuclear and heteronuclear couplings, T_1 and T_2 relaxation was 0.24%.

Diffusion ordered-qNMR spectroscopy (DOSY-qNMR) – In DOSY, NMR peaks of the interfering substances can be separated from those of the anlayte based on the diffusion coefficient under a gradient magnetic field. Cao et al. quantified sucrose in beverages containing various types of sugars.[50] Cellobiose, which has a similar diffusion coefficient to that of sucrose, was used as IS. A linear response was observed between sucrose concentration and the peak area ratio of the analyte and IS. The

DOSY-qNMR results were comparable with those obtained from the enzymatic F-kit assay and HPLC.

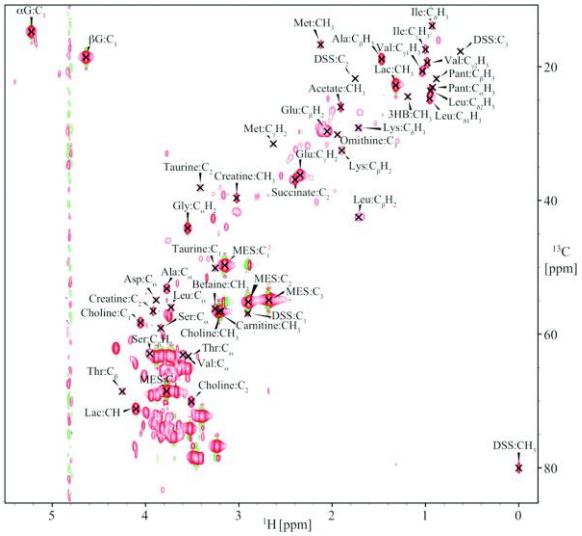

Figure 2. HSQC$_1$ spectrum of bovine liver extract. Adapted with permission from Ref. 48.

Conclusions

Purity assessment of a high-purity material is a critical step in establishing the SI-traceability, and NMR is considered as one of the primary methods in purity assessment. As qNMR is complementary to the mass balance method, the reliability of the purity assay can be increased by combining the two methods. Although the qNMR procedure is simple, experiments should be carried out under optimum conditions to obtain accurate results. QNMR has a few limitations including peak overlap and, low concentration sensitivity. Novel techniques will be developed continuously to solve the drawbacks of qNMR.

Acknowledgements

This research was supported by the Korea Research Institute of Standards and Science under the project ‘Establishing measurement standards for organic analysis (grant number 20011034)’.

References

1. P. De Bièvre, R. Dybkær, A. Fajgelj, and D. B. Hibbert, *Pure Appl. Chem.*, **83**, 1873. (2011)
2. S. Westwood, T. Choteau, A. Daireaux, R. D. Josephs, and R. I. Wielgosz, *Anal. Chem.*, **85**, 3118. (2013)
3. S. R. Davies, M. Alamgir, B. K. H. Chan, T. Dang, K. Jones, M. Krishnaswami, Y. W. Luo, P. S. R. Mitchell, M. Moawad, H. Swan, and G. J. Tarrant, *Anal. Bioanal. Chem.*, **407**, 7983. (2015)
4. B. M. Huang, S. Y. Xiao, T. B. Chen, Y. Xie, P. Luo, L. Liu, and H. Zhou, *J. Pharm. Biomed. Anal.*, **139**, 193. (2017)
5. G. del Campo, J. Zuriarrain, A. Zuriarrain, and I. Berregi, *Food Chem.*, **196**, 1031. (2016)
6. B.-M. Huang, S.-Y. Xiao, T.-B. Chen, Y. Xie, P. Luo, L. Liu, and H. Zhou, *J. Pharm. Biomed. Anal.*, **139**, 193. (2017)
7. G. F. Pauli, B. U. Jaki, and D. C. Lankin, *J. Nat. Prod.*, **68**, 133. (2005)
8. G. K. Webster and S. Kumar, *Anal. Chem.*, **86**, 11474. (2014)
9. T. Saito, T. Ihara, M. Koike, S. Kinugasa, Y. Fujimine, K. Nose, and T. Hirai, *Accred. Qual. Assur.*, **14**, 79. (2009)
10. K. A. Lippa, D. L. Duewer, M. A. Nelson, S. R. Davies, and L. G. Mackay, *Accred. Qual. Assur.*, **24**, 407.

(2019)
11. T. Saito, T. Yamazaki, and M. Numata, *Metrologia*, **56**, 054002. (2019)
12. T. Huang, H. Li, W. Zhang, M. Numata, L. Mackay, J. Warren, H. Jiao, S. Westwood, and D. Song, *Metrologia*, **57**, 014004. (2020)
13. T. Saito, T. Ihara, T. Miura, Y. Yamada, and K. Chiba, *Accred. Qual. Assur.*, **16**, 421. (2011)
14. R. J. Wells, J. Cheung, and J. M. Hook, *Accred. Qual. Assur.*, **9**, 450. (2004)
15. T. Rundlöf, M. Mathiasson, S. Bekiroglu, B. Hakkarainen, T. Bowden, and T. Arvidsson, *J. Pharm. Biomed. Anal.*, **52**, 645. (2010)
16. R. Rigger, A. Rück, C. Hellriegel, R. Sauermoser, F. Morf, K. Breitruck, and M. Obkircher, *J. AOAC Int.*, **100**, 1365. (2017)
17. M. A. Nelson, J. F. Waters, B. Toman, B. E. Lang, A. Rück, K. Breitruck, M. Obkircher, A. Windust, and K. A. Lippa, *Anal. Chem.*, **90**, 10510. (2018)
18. S. Westwood, T. Yamazaki, T. Huang, B. Garrido, I. Ün, W. Zhang, G. Martos, N. Stoppacher, T. Saito, and R. Wielgosz, *Metrologia*, **56**, 064001. (2019)
19. C. H. Cullen, G. J. Ray, and C. M. Szabo, *Magn. Reson. Chem.*, **51**, 705. (2013)
20. G. K. Jayaprakasha and B. S. Patil, *Talanta*, **153**, 268. (2016)
21. I. W. Burton, M. A. Quilliam, and J. A. Walter, *Anal. Chem.*, **77**, 3123. (2005)
22. L. Barantin, A. L. Pape, and S. Akoka, *Magnetic Resonance in Medicine*, **38**, 179. (1997)
23. S. Akoka, L. Barantin, and M. Trierweiler, *Anal. Chem.*, **71**, 2554. (1999)
24. V. Silvestre, S. Goupry, M. Trierweiler, R. Robins, and S. Akoka, *Anal. Chem.*, **73**, 1862. (2001)
25. K. Mehr, B. John, D. Russell, and D. Avizonis, *Anal. Chem.*, **80**, 8320. (2008)
26. R. D. Farrant, J. C. Hollerton, S. M. Lynn, S. Provera, P. J. Sidebottom, and R. J. Upton, *Magn. Reson. Chem.*, **48**, 753. (2010)
27. T. Yamazaki, S. Nakamura, and T. Saito, *Metrologia*, **54**, 224. (2017)
28. R. J. Abraham, J. J. Byrne, L. Griffiths, and M. Perez, *Magn. Reson. Chem.*, **44**, 491. (2006)
29. N. Saito, T. Saito, T. Yamazaki, Y. Fujimine, and T. Ihara, *Accred. Qual. Assur.*, **22**, 171. (2017)
30. T. Saito, S. Nakaie, M. Kinoshita, T. Ihara, S. Kinugasa, A. Nomura, and T. Maeda, *Metrologia*, **41**, 213. (2004)
31. P. A. Hays and T. Schoenberger, *Anal. Bioanal. Chem.*, **406**, 7397. (2014)
32. F. Malz and H. Jancke, *Anal. Bioanal. Chem.*, **385**, 760. (2006)
33. T. Huang, W. Zhang, X. Dai, N. Li, L. Huang, C. Quan, H. Li, and Y. Yang, *Anal. Methods*, **8**, 4482. (2016)
34. W. Zhang, T. Huang, H. Li, X. Dai, C. Quan, and Y. He, *Talanta*, **172**, 78. (2017)
35. M. Godejohann, A. Preiss, and C. Mügge, *Anal. Chem.*, **70**, 590. (1998)
36. T. Saito, R. Iwasawa, T. Ihara, S. Kinugasa, A. Nomura, and T. Maeda, *Chromatography*, **24**, 117. (2003)
37. J. Sýkora, P. Bernášek, M. Zarevúcká, M. Kurfürst, H. Sovová, and J. Schraml, *J. Chromatogr. A*, **1139**, 152. (2007)
38. T. Vehovec and A. Obreza, *J. Chromatogr. A*, **1217**, 1549. (2010)
39. G. K. Webster, I. Marsden, C. A. Pommerening, C. M. Tyrakowski, and B. Tobias, *J. Pharm. Biomed. Anal.*, **49**, 1261. (2009)
40. Y. Kitamaki, N. Saito, T. Yamazaki, S. Otsuka, S. Nakamura, Y. Nishizaki, N. Sugimoto, M. Numata, and T. Ihara, *Anal. Chem.*, **89**, 6963. (2017)
41. R. D. Josephs, N. Stoppacher, A. Daireaux, T. Choteau, K. A. Lippa, K. W. Phinney, S. Westwood, and R. I. Wielgosz, *TrAC, Trends Anal. Chem.*, **101**, 108. (2018)
42. J. E. Melanson, M.-P. Thibeault, B. B. Stocks, D. M. Leek, G. McRae, and J. Meija, *Anal. Bioanal. Chem.*, **410**, 6719. (2018)

43. P. Giraudeau, *Magn. Reson. Chem.*, **52**, 259. (2014)
44. S. Heikkinen, M. M. Toikka, P. T. Karhunen, and I. A. Kilpeläinen, *J. Am. Chem. Soc.*, **125**, 4362. (2003)
45. D. J. Peterson and N. M. Loening, *Magn. Reson. Chem.*, **45**, 937. (2007)
46. H. Koskela, O. Heikkilä, I. Kilpeläinen, and S. Heikkinen, *Journal of Magnetic Resonance*, **202**, 24. (2010)
47. K. Hu, W. M. Westler, and J. L. Markley, *J. Am. Chem. Soc.*, **133**, 1662. (2011)
48. K. Hu, J. J. Ellinger, R. A. Chylla, and J. L. Markley, *Anal. Chem.*, **83**, 9352. (2011)
49. F. Fardus-Reid, J. Warren, and A. LeGresley, *Anal. Methods*, **8**. (2016)
50. R. Cao, A. Nonaka, F. Komura, and T. Matsui, *Food Chem.*, **171**, 8. (2015)