Evaluating Sampling Uncertainty in the Quantitative ^{1}H Nuclear Magnetic Resonance Analysis of Lignin

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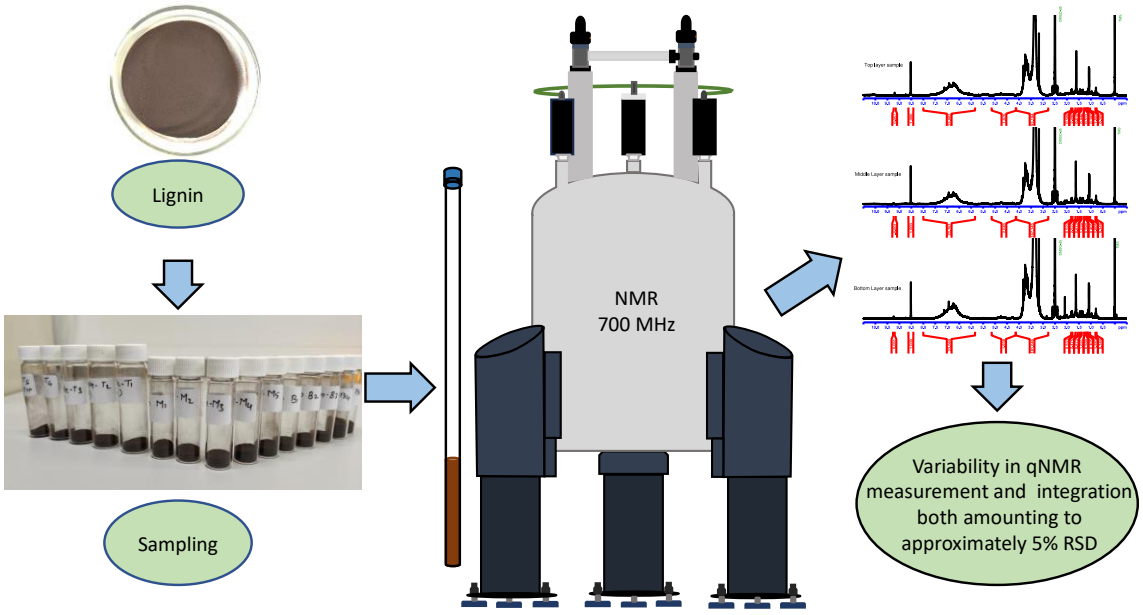
GRAPHICAL ABSTRACT



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In recent years, lignin analysis utilizing quantitative nuclear magnetic resonance (qNMR) has attracted considerable interest and has been the subject of numerous studies. However, evaluating the measurement uncertainty of qNMR results of lignin remains a challenge. Specifically, uncertainty originating from lignin sampling or subsampling has been overlooked in a large majority of articles. Although lignin is a reasonably homogeneous substance, it is nevertheless a solid, and individual samples collected from the same bulk may have somewhat different compositions depending on mixing and the amount of sample taken. The objective of this study was to evaluate the influence of sampling uncertainty on qNMR analysis of lignin-based analysis as a case study, with an exclusive focus on the relative quantification method. The results from this study demonstrate that sample-to-sample variations can contribute to approximately half of the variability in actual qNMR measurements. The relative standard deviation (RSD) of sample-to-sample variability was 2.4%. In contrast, the other sources of variability related to qNMR, including measurement, baseline irregularities, and partial peak overlap, caused an RSD of 4.4%. The total variability RSD was 5.0%. In this article, two calculation approaches were presented for evaluating the uncertainty due to sampling from replicate measurement data of different samples, which may be helpful for practitioners in the field.

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Keywords: Lignin; NMR; Quantitative NMR; Sampling; Uncertainty

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INTRODUCTION

Lignin is the second-most abundant type of biopolymer on Earth, accounting for 30% of organic carbon (Boerjan *et al.* 2003; Lu *et al.* 2017; Pawade *et al.* 2023). Lignin is obtained in large amounts (approximately 60 to 70 million tonnes annually) as a by-product of the paper and pulp industry. As such, it is one of the most important renewable organic feedstocks. However, lignin is currently significantly underutilized compared with cellulose (An *et al.* 2015). Lignin is one of the few renewable aromatic biopolymers and stands out for its aromatic nature among other biopolymers (Wang *et al.* 2021). Approximately 98% of lignin is currently used as a source of energy and heat (Constant *et al.* 2016).

Based on the plant species, tissue type, and specific cell wall layer, lignin is composed of aromatic units with different structures and ratios of aromatic units (Happs *et al.* 2021). In general, lignin consists primarily of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. The S, G, and H units interlink with each other *via* different linkers/bonds: β-O-4 (β-ether), α-O-4, β-β (resinol), and β-5 (Balakshin *et al.* 2011). β-O-4,

β-β, β-5, PhOMe, PhOH, aliphatic methoxy and aliphatic OH, aromatic H, and aldehyde substructures and linkages are the prevalent structural fragments that are typically quantitatively analyzed (Balakshin *et al*. 2011; Shimizu *et al*. 2017). Analytical techniques can determine the types of monolignols and their contents, the distribution of inter-unit linkages, and the functional groups that make up the chemical structure of lignin. For advanced applications of lignin, regardless of the depolymerization approach, fundamental knowledge of its structural features and physicochemical properties is essential (Nayak *et al*. 2020).

Quantitative nuclear magnetic resonance (qNMR) spectrometry seems to be the most widespread and versatile technique for the quantitative analysis of lignin (Capanema *et al*. 2004, 2005). It enables determining numerous structural characteristics of lignin by using ^{1}H (Faix *et al*. 1994), ^{13}C (Balakshin and Capanema 2015a,b; Balakshin *et al*. 2016), or ^{31}P (Gracia-Vitoria *et al*. 2022) (after derivatization) as the nuclei, as well as different two-dimensional techniques (Zhang and Gellerstedt 2007; Constant *et al*. 2016; Amiri *et al*. 2019). The authors recently developed an interest in determining the possible accuracy attainable through the qNMR analysis of lignin, and the authors conducted a literature survey (Pawade *et al*. 2023). The literature analysis revealed several uncertainty sources inherent in the measurement, for example, the repeatability of spectra (especially if the signal-to-noise ratio is low), the accuracy of peak integration (especially in the case of baseline irregularities), an overlap of signals of interest with other signals (Balakshin and Capanema 2015), and different NMR-specific uncertainty sources, such as deviations in the coupling constant, resonance offset effects, and effects of ^{1}H T_1 relaxation (Zhang and Gellerstedt 2007; Amiri *et al*. 2019).

Interestingly, however, very little attention has been devoted to the possible uncertainty arising from the sampling or subsampling of the lignin. In addition, the sampling protocol used has not been reported in most publications. At the same time, it is widely recognized that sampling (or subsampling) is among the most crucial uncertainty sources in most chemical analyses (Ramsey *et al*. 2019; Medeiros *et al*. 2022; Sano and Lourenço 2023).

Although lignin can be reasonably homogeneous, it is still a natural solid material, and such materials are always, at least to some extent, inhomogeneous. This can be caused, *e.g.*, by raw material variability (powder composition can change during the fill run), segregation (smaller particles tend to move to the bottom, and larger particles tend to stay on top), or absorption of moisture (topmost layer will absorb more moisture than the middle or bottom layers). Thus, subsamples taken from the same bulk of lignin can have somewhat different compositions and, therefore, give different NMR spectra.

Uncertainty from sampling was mentioned only in a couple of reports (Froass *et al*. 1998; Balakshin and Capanema 2015), and only in one of them was some quantitative data presented (Froass *et al*. 1998). This information enabled the authors to approximate the subsampling and sample preparation variability, which ranged from 5% to 8% relative standard deviation (RSD). At the same time, in most reports, even the most in-depth ones, sampling/subsampling is not mentioned. As a result, in many cases, it is not known whether replicate measurements were performed using the same or different samples/subsamples. In such cases, when an RSD estimate between replicate measurements is provided, its meaning remains obscure.

Considering this, the authors decided to investigate the uncertainty due to the inhomogeneity of subsampling in the analysis of commercial lignin and how it compares with the variability of NMR spectrometric analysis itself.

EXPERIMENTAL

Reagents and Samples

Softwood kraft lignin (alkali, low sulfate content) from pine wood was procured from Sigma-Aldrich USA, and DMSO-d_6 (99.8% with 0.03% tetramethylsilane (TMS)) was acquired from Deutero GmbH Kastellaun, Germany.

Sampling and Sample Preparation

The sampling protocol was as follows: Lignin samples were collected from various parts of a lignin container containing 1.0 kg of lignin. Altogether, 15 samples were obtained from the bulk: 5 from the top layer, 5 from the middle layer, and 5 from the bottom layer. Every sample was analyzed in quadruplicate from the same solution over a time span of up to a little more than a week. To prepare the samples for ^{1}H nuclear magnetic resonance (NMR) analysis, each sample was weighed to 5.0 mg of lignin in an NMR tube and dissolved into 0.6 g DMSO-d_6. A small sample size and low concentration of the resulting solution were used to ensure the complete dissolution of lignin. A larger sample size of a larger lignin sample was not used for three reasons: (1) complete dissolution – very important to guarantee good-quality spectra – would have required large amounts of the expensive deuterated solvent; (2) in many investigations, in contrast to this work, only small samples may be available; and (3) in this work the uncertainty of weighing does not influence the results, as all the quantification is done using ratios of peak areas. The NMR tube was firmly closed and held in an ultrasonic bath for 15 min to completely dissolve the lignin residue. All samples were prepared using the same method within 2 to 3 days. The ^{1}H NMR spectra of each sample were measured four times, and replicate analyses were performed in random order within a two-week period.

NMR Spectrometry

Proton nuclear magnetic resonance (^{1}H NMR) spectrometry was used in this work. All spectra were acquired with a Bruker Avance-III 700 MHz NMR spectrometer from Switzerland with a 5-mm BBO (broadband observe) probe. The sample temperature was maintained at 25 °C for all measurements. The ^{1}H NMR spectra were acquired using 81920 data points, 30° pulse, recycle time of 5.91 s (acquisition time 2.9 s, relaxation delay 3.0 s) (Zhang and Gellerstedt 2007), and 2048 scans preceded by 4 steady-state scans.

For reference, the authors measured T_1 values and obtained 1.0 to 2.0 s, depending on the specific structural fragment. Thus, it is reasonable to argue that the chosen NMR acquisition parameter (mainly the D_1 value) may have been insufficient to obtain full relaxation of signals between scans, and therefore, signal areas can be biased. However, absolute quantification was not the focus of this study. All results and conclusions are based on the between-sample variability of the ratios of integrals. This information can still be obtained because the signal inaccuracies either cancel out or remain the same for replicate measurements.

All acquired ^{1}H NMR spectra were subjected to the same data treatment using TopSpin software (Bruker TopSpin 3.2). The spectra were zero-filled to 256,000 points, line broadening (LB) of 0.1 Hz was used, Fourier transformation, manual phase correction, and baseline correction was automatically done using the spline baseline correction method (the high importance of accurate baseline correction has been stressed in literature) (Balakshin and Capanema 2015a,b). The spline baseline correction was based on a predefined set of data points, which were considered part of the baseline. The authors chose

the same baseline points (31 points, which were saved in a *baslpnts* file that was used for spline baseline correction on every spectrum) on every spectrum to define the baseline. The TopSpin software then was used to fit the regions between these points and subtract from the measured spectrum (the command for this procedure in the TopSpin software is *sab*).

All the ^{1}H NMR spectra were calibrated using the signal corresponding to TMS at δ = 0.0 ppm. Then, all signal regions of the spectra were integrated, excluding the TMS and DMSO-d_5 signals. A certified NMR reference standard by Bruker was routinely used to calibrate the 90° ^{1}H pulse, and the Prosol table (where the pulse lengths were stored) was updated regularly.

All quantitative analyses were performed using a reference peak; that is, all the peak intensity ratios in all regions, both aromatic and aliphatic, were calculated relative to the integral of this peak. The reference peak should be strong and well-separated. Two such peaks were identified: one in the region of 8.40 to 8.65 ppm, belonging to a formate salt in the lignin product, and another one in the region of 1.56 to 1.60 ppm, belonging to aliphatic fragments. Data analysis was performed separately with these two reference peaks. The signals or signal ranges that were used for integration were the aldehyde peak (9.18 to 9.30 ppm), aromatic region (5.80 to 8.00 ppm), peak related to O-CH/O-CH$_2$ (4.13 to 5.10 ppm), methoxy and hydroxy peaks, and residual water peak from DMSO solvent (2.80 to 4.10 ppm). The signals at 0.60 to 1.50 ppm were due to aliphatic protons that were not oxygenated (An *et al*. 2015). From all samples, 11 different signal intensity ratios, listed in Table 1, were quantified (both peaks that were used as reference peaks were quantified only when the other peak was the reference). The peaks and ranges, as well as their identifiers, are shown in Fig. 1.

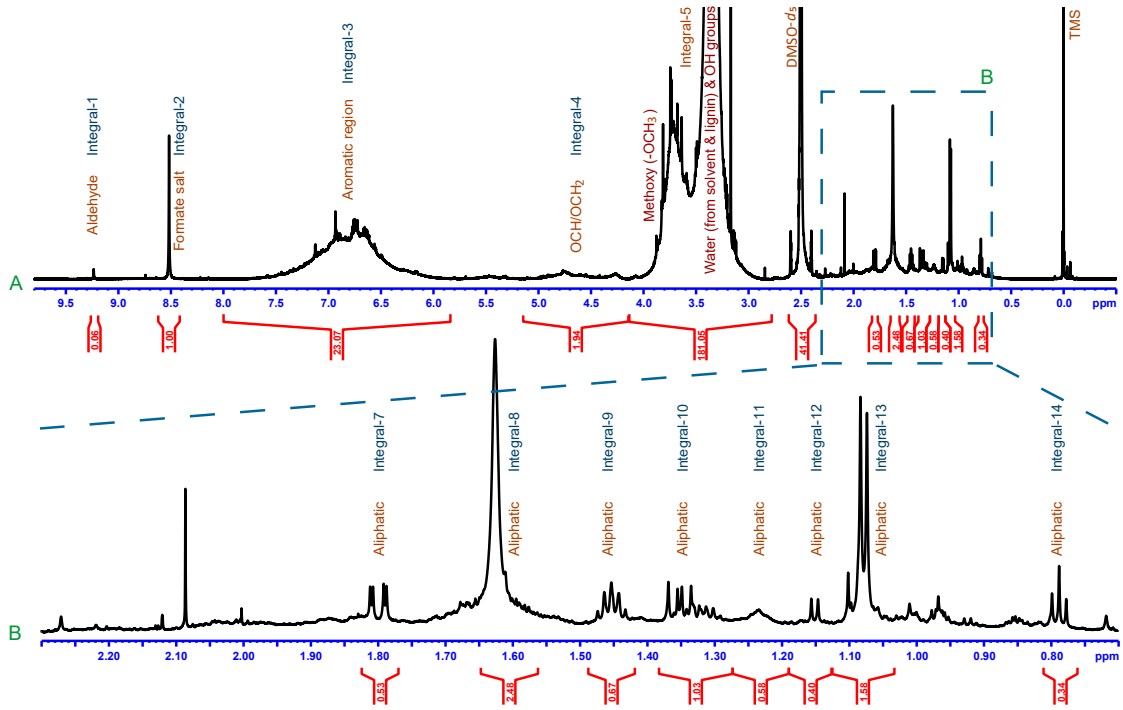

Fig. 1. (A) Representative example of an acquired ^{1}H NMR spectrum of kraft lignin sample in DMSO-d_6, (B) Aliphatic region from 0.70 to 2.30 ppm; See the Supporting Information for more spectra

To reveal the effect of sample preparation on the variability of integrals, as opposed to NMR measurement and integration, such signals and ranges were selected for further analysis that were sufficiently (but not overly) intense and where satisfactory baseline correction was possible. For this reason, the region corresponding to methoxy and hydroxy (2.80 to 4.10 ppm) groups were excluded (very high intensity of the signal and residual water involved, making it difficult to correct the baseline) as well as the peak in the region of 9.18 to 9.30 ppm due to its very low intensity.

Data Analysis

The data obtained were checked for outliers using the Dixon Q test. The Dixon Q test was carried out at 95% confidence level (without p-value adjustment), separately for every type of integral for each type of sample (top, middle, bottom), pooling together the data of the 5 replicates. This resulted in 30 Dixon tests, each with 20 datapoints. To check the normality of the data, the data of all integrals were normalized, and the resulting normalized datasets were pooled into a 600 data point set. The normality of the distribution was evaluated by visual comparison with the cumulative normal distribution curve, as well as the linearized normality plot (see the Excel file in the Supporting Information).

Data analysis was performed with the aim of dissecting the overall variability of the relative peak intensities into two components: Variability from measurement and variability from sampling. Data analysis was performed separately for each of the 10 intensity ratios. Two approaches were used. One was the analysis of variance (ANOVA) approach, described in detail in Van Der Veen and Pauwels (2000).

The other approach (termed as "RSD approach") looks at the overall variability of the results as relative standard deviation (RSD_{Total}), which can be regarded as composed of two components: Variability caused by sampling (RSD_{Sampling}) and variability caused by measurement (RSD_{Meas}):

$$RSD_{\text{Total}} = \sqrt{RSD_{\text{Sampling}}^2 + RSD_{\text{Meas}}^2} \quad (1)$$

Sampling variability cannot be directly evaluated because any evaluation of variability always involves measurements. Therefore, an indirect approach was used, as follows: The variability found as standard deviation over the results obtained with all samples taken from the bulk material (RSD_{Total}) includes variabilities originating from sampling as well as variabilities from measurement. Variability found from replicate measurements with the same sample (RSD_{Meas}) includes the variability of measurement only. This can be expressed by the following Eq. 2:

$$RSD_{\text{Sampling}} = \sqrt{RSD_{\text{Total}}^2 - RSD_{\text{Meas}}^2} \quad (2)$$

For every individual signal intensity ratio, the RSD_{Total} was determined as the RSD of the individual intensity ratios of all replicates of all 15 samples. The RSD_{Meas} for every individual signal ratio was obtained as pooled RSD (for explanations, see section 6 of the course presented in Leito, Helm, and Jalukse (2015) of the 4 replicate measurement results of the same signal intensity ratio from the 15 samples. The RSD_{Sampling} for each individual signal ratio was obtained from Eq. 2.

To obtain an estimate of the average RSD across the signal ratios corresponding to the different peaks, the individual RSD values were pooled by calculating the root mean square (RMS) of the RSD values ("Pooled RSD values" in Table 1).

RESULTS AND DISCUSSION

The outlier check revealed one outlier data point out of a total of 600. The authors did not find the reason for the deviation and accordingly did not consider it justified to eliminate it because (1) the outlier tests were carried out at a 95% confidence level, which in the case of 30 tests means that there is a probability of $1 - 0.95^{30} = 0.79$ that there is at least one outlier and (2) given that the overall amount of data is large and leaving the data point out would not change anything in the conclusions of this work. The overall cumulative distribution of the data was indistinguishable from the normal distribution (see Supporting Information).

As expected, the two data analysis approaches yielded almost identical results. There was a somewhat more difference between the results obtained with the different reference peaks. The RMS averages of all four sets of results (two data-analysis approaches and two reference peaks) are presented in Table 1. A detailed calculation file containing the calculations, all individual results obtained with both approaches, and both reference peaks is provided in the Supporting Information. The ANOVA approach may be considered fundamentally more rigorous. At the same time, the RSD approach gives a number of RSD values as interim results, which are more easily interpretable than the interim quantities of ANOVA and may be usable by themselves, for example, for tracking shortcomings in experiments.

Table 1. Overall Relative Standard Deviations of Signal Ratios (RSD_{Total}), RSD due to measurement (RSD_{Meas}), and RSD due to sampling ($RSD_{Sampling}$)

Signal Integrals Used for Calculating the Ratios against Signal at 8.40 to 8.65 ppm (Formate Salt Integral-2)	RSD_{Total}	RSD_{Meas}	$RSD_{Sampling}$
		Individual RSD Values	
Aldehyde Integral-1 (9.18 to 9.30 ppm)	3.5%	2.7%	2.3%
Aromatic Region Integral-3 (5.80 to 8.00 ppm)	4.8%	3.4%	3.4%
O-CH/O-CH_2 Integral-4 (4.13 to 5.10 ppm)	7.4%	6.3%	3.9%
Integral-7 (1.77 to 1.79 ppm)	3.7%	3.1%	2.0%
Integral-8 (1.56 to 1.60 ppm)	3.5%	2.7%	2.2%
Integral-9 (1.40 to 1.45 ppm)	4.5%	4.0%	2.1%
Integral-10 (1.26 to 1.32 ppm)	4.5%	4.1%	1.7%
Integral-11 (1.19 to 1.23 ppm)	5.7%	5.5%	1.6%
Integral-12 (1.12 to 1.15ppm)	6.7%	6.4%	1.9%
Integral-13 (1.03 to 1.08 ppm)	3.3%	2.8%	1.8%
Integral-14 (0.76 to 0.79 ppm)	5.4%	5.0%	1.9%
		Pooled RSD Values	
	5.0%	4.4%	2.4%

Data presented as RMS averages of 2 data analysis approaches and 2 different reference peaks. See the Supplementary Information using the weblink provided in the Appendix for the complete calculation and individual results.

As shown in Table 1, the RSD_{Total}, RSD_{meas}, and $RSD_{sampling}$ values varied from 3.3% to 7.4%, 2.7% to 6.4%, and 1.6% to 3.9%, respectively. In most cases, RSD_{Meas} was larger than $RSD_{Sampling}$. There did not seem to be any clear pattern in these variabilities, nor are there any significant outliers. Therefore, it is reasonable to assume that the observed differences were caused by statistical fluctuations.

The pooled RSD values demonstrate that under the experimental conditions used, the RSD values of NMR measurement (together with peak integration) and sampling to the overall RSD of the measurement results differ by approximately two times. This means that uncertainty due to sampling is by no means a negligible source of uncertainty in this type of analysis and, in contrast to general practice (Pawade *et al*. 2023), should always be considered.

One of the challenges in the measurement was the poor separation of some signals. In addition, baseline correction represents an important difficulty in data processing if quantitative results are desired. In the replicate measurements of lignin, there were minor differences in the peak shapes and slight changes in the chemical shifts. However, by using a larger number of scans, it was possible to address such issues. This technique allows the calculation of the sampling uncertainty of various types of lignin.

While studies of effects of NMR-related parameters on the accuracy of quantitative analysis of lignin are numerous, this is essentially the first study to examine uncertainty/variability due to sampling, and only one material type was examined. In future studies, it might be interesting to investigate the variability due to the sampling of lignins obtained using different technologies and possibly other natural materials. Additionally, the lignin used in this study was relatively homogeneous, being a commercial product in the form of a fine powder. In contrast, samples obtained from pulp mills or industrial sources are likely to exhibit higher inhomogeneity and thus higher sampling uncertainty compared to the results obtained in this study.

CONCLUSIONS

1. The results demonstrated that under the experimental conditions, the uncertainty due to the sample-to-sample variability was approximately half of the uncertainty accounting for the variability in qNMR measurement and integration. Thus, sampling/subsampling is, by all means, an important uncertainty source.
2. Two additional aspects are worth mentioning. The sample size was at the low end of what is typically used for such an analysis. Thus, by using larger samples, it may be possible to observe smaller sample-to-sample variability. However, this experimental setup and the results presented illustrate a "good case," as the sample was a commercial product from an established industrial process, which can be assumed to be reasonably well mixed. For this reason, the sample-to-sample variability found in this study may not necessarily be applicable in more exploratory situations, such as the analysis of less homogeneous crude products or products from experimental processes.
3. The presented calculation file in Supporting Information can serve as a template for practitioners interested in evaluating the uncertainty due to sampling from replicate measurement data of different samples.

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APPENDIX

Additional spectra, as well as the file containing all the integral values and all calculations (in the MS Excel format), are available as Supporting Information at https://doi.org/10.6084/m9.figshare.25516477