A community-built calibration system: The case study of quantification of metabolites in grape juice by qNMR spectroscopy

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ABSTRACT: Nuclear Magnetic Resonance (NMR) is an analytical technique extensively used in almost every chemical laboratory for structural identification. This technique provides statistically equivalent signals in spite of using spectrometer with different hardware features and is successfully used for the traceability and quantification of analytes in food samples. Nevertheless, to date only a few internationally agreed guidelines have been reported on the use of NMR for quantitative analysis. The main goal of the present study is to provide a pipeline to assess the reproducibility of NMR data produced for a given matrix by spectrometers from different manufacturers, with different magnetic field strengths, age and hardware configurations. The results have been analysed through a sequence of chemometric tests to generate a *community-built calibration system* which was used to verify the performance of the spectrometers and the reproducibility of the predicted sample concentrations.

INTRODUCTION

Relative or absolute quantification of an analyte in a matrix consists of a sequence of operations carried out under defined and agreed methods, which are developed according to technical specificity of the analytical tool, the analyte and the matrix. Among the operations required to quantify a substance, calibration processes deserve special consideration. According to the International Bureau of Weights and Measures, calibration is defined as "Operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties (of the calibrated instrument or secondary standard) and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication".[1] Usually, a calibration curve is developed to find the optimal equation correlating the response of the selected analytical technique and the concentration of a set of standard samples of the analyte with known concentration. Such equation is used to derive the concentration of the analyte contained in an unknown sample. The calibration conditions vary with time and instrumental use thus, depending on the specific analytical issue, a periodical update is required. Therefore, the calibration process has an important impact on both cost and execution time of the analysis.

Establishing metrological traceability is a prerequisite to obtain a reliable metrological comparability of the measurement results produced at different laboratories and at different times. As stated by De Bièvre et al., achieving metrological comparability of measurement results requires the definitions of concepts of calibration hierarchies providing metrological traceability chains, which enable the establishment of metrological traceability of measured quantity values to a common and stable metrological reference.[2] Ideally, in order to produce reliable and traced measurement results with the corresponding measurement uncertainties for a given method, the whole analytical pipeline should be based on a community-built system able to simultaneously manage calibration and traceability steps for many operators. Such a system should *i*) collect calibration data produced by many operators, *ii*) process data to develop a community-built reference calibration curve and *iii*) provide results (exploiting the community-built reference calibration curve) to many operators after submission of data regarding unknown samples. Many advantages may derive from using such system. First, a number of community-built reference calibration curves and, thus, reference concentration values for different analytes in different

matrices can be developed. Moreover, such community-built database could be continuously made more robust by introduction of new calibration data, produced also by laboratories not directly involved in the initial calibration curve building. Therefore, the reference calibration data (curve parameters and predicted analyte concentration) will become more precise and accurate and could be ultimately used as reference values to test the performance of the laboratories.

The only requirement for creating this community-built system is the use of an analytical technique able to generate, for a given sample, statistically equivalent signals. In other words, any sample should produce the same instrumental response when analysed by different instruments.

In this context, recently, by means of interlaboratory comparisons (ILCs), we demonstrated that NMR spectroscopy can provide statistically equivalent signals when the same sample is analysed by spectrometers that are different in terms of magnetic field strength, manufacturer, hardware configurations and age.[3,4] Indeed, the exclusive correlation between the resonance frequency of a signal and the type of nuclei associated to that signal, makes NMR spectroscopy a powerful technique for structural determination and quantification.[5,6] Since the area of a NMR signal is linearly proportional to the number of NMR active nuclei generating the signal, the response factor (ratio between the signal produced by the analyte and the quantity of analyte which produces the signal) is independent of the molecule and the analyte quantification can be achieved directly by calculating integral of the NMR signal.[7] Moreover, the design of new pulse sequences for FIDs acquisition[8–13] and novel algorithms for data processing[14–17] enhanced the capability of NMR for discriminating among very similar compounds contained in complex mixtures, as pharmaceutical, natural products, agrochemicals, foodstuff, and biofluids. Nevertheless, to date few official protocols have been reported which employ NMR technique for purity assessment and quantification purposes.[18] While the experimental conditions (pulse sequence, acquisition parameters, post-processing strategy) assuring the intra-laboratory repeatability are well established,[19–21] still few studies are available that discuss the reproducibility assessment of quantitative NMR (qNMR) data obtained when the same sample is analysed by different operators and/or by spectrometers with variable features (manufacturer, B_0 field strength).[22–25] Taking into consideration the extensive application of qNMR in different fields of chemical science, it appears as a matter of urgency to overcome this significant shortcoming and make qNMR an internationally accepted standard analytical technique.

In this paper, we provide a pipeline to assess the reproducibility of NMR data produced for a given matrix by spectrometers from different manufacturers, with different magnetic field strengths, age and hardware configurations. Moreover, we introduce a community-built quantification system able to perform quantitative analysis and to assess performance of the laboratories. The aspects affecting the interlaboratory reproducibility are also discussed. Specifically, by exploiting the big amount of spectroscopic data produced during an interlaboratory comparison involving 65 spectrometers from 12 countries, the concentrations of four selected metabolites (alanine, arginine, glucose, and fructose) contained in the grape juice (*cv.* Primitivo) are predicted *via* calibration lines developed by standard addition method. A sequence of appropriate chemometric tests (Figure 1, model development) are applied to the predictive models developed individually by the ILC participants. Upon evaluation of the likely sources of error, it was established a strategy for assessing the performance of the laboratories during the different stages of the quantitative analysis. The well-performing models were tested to predict the unknown concentrations of the metabolites contained in a test sample and the obtained data were evaluated in terms of reproducibility, allowing for the identification and validation of statistical equivalent signals in the NMR spectrum (Figure 1, performance assessment).

Figure 1 here

Figure 1. Schematic representation of the selection process for the evaluation of the prediction models designed by 65 ILC participants.

EXPERIMENTAL SECTION

Materials. 3-(Trimethylsilyl)-2,2,3,3-tetradeutero-propionic acid sodium salt (TSP, CAS No. 24493-21-8, 99 %D, Armar Chemicals, Döttingen, Switzerland), sodium azide (NaN_3, CAS No. 26628-22-8; ≥99.5%, Sigma-Aldrich, Milan, Italy), deuterium oxide (D_2O, CAS. N. 7789-20-0, 99.86 %D, Eurisotop, Saclay, France) and methanol-d_4 (CD_3OD, CAS. No. 811-98-3, 99.80 %D, Eurisotop, Saclay, France) were used for sample preparation. NMR tubes (Norell 509-UP 7) were provided by Norell, Landisville NJ, US. The NMR samples were prepared using an automated system for liquid handling (SamplePro Tube, Bruker BioSpin).

Wine grape samples (*cv*. Primitivo; Centro di Ricerca, Sperimentazione e Formazione in Agricoltura "Basile-Caramia (CRSFA), Locorotondo, Bari, Italy) were collected according to official recommendations (Regulations (CE) no. 834/2007, no. 889/2008, no. 1235/2008 and following modifications). 50 samples of *cv*. Primitivo were collected as follows: 30 berries were harvested randomly from different parts of the same plant for each sample. The samples were labelled according to the plant of origin, which was marked with a number and a letter, indicating respectively the vine-row and the sector of the vine-row to which the plant belonged. One larger sample (1 Kg) was collected randomly from three plants belonging to the vineyard and labelled according to the same procedure. The samples were refrigerated at 4°C and transferred from the field to the laboratory, where they were stored at –20°C.

Experimental procedure. The interlaboratory comparison was organized according to EN ISO/IEC 17043:2010 and reference normative therein (Conformity assessment - General requirements for proficiency testing)[26] with 52 registered participants and 75 available spectrometers.[27] 75 sets of flame-sealed NMR tubes were delivered to the participants and 65 spectrometers [300 (2), 400 (22), 500 (18), 600 (18) and 700 (5) MHz; manufacturers: Bruker (52), Varian/Agilent (9) and Jeol (4)] returned NMR data. Each set of NMR tubes (labelled as T, A, B, C, D, E, X) included a sample containing *cv.* Primitivo grape juice (tube X), and five test tubes (A-E) containing spiked solutions of four metabolites naturally contained in the grape juice (glucose, fructose, arginine and alanine). Tube T contained pure methanol-d_4 (CD_3OD, 99.80 %D) whose residual ^1H signals were used as an NMR thermometer in order to calibrate the temperature of each spectrometer at 298.1 ± 0.1 K.[28] Tube X, containing aqueous solutions of wine grape juice (*cv*. Primitivo), was prepared as follows: 10 berries were defrosted at room temperature for 60 minutes. They were mechanically pressed and the resulting grape juice (~ 5 mL) was centrifuged (Ettich Rotofix 32A, 2500 g, 15 minutes). The supernatant (1.08 mL) was combined with a solution (84.6 mg / 50 mL) of NaN_3 in buffer [$(HC_2O_4)^-/(C_2O_4)_2^-$ 0.11 M, pH 4.2], giving Solution M1. By means of a robotic system for liquid handling tasks (Bruker Sample Pro), 318 μl of solution M1 was combined stepwise with a volume of the buffer solution (222 μl) and a volume of a TSP/D_2O solution (60 μL, 0.10 g of TSP in 50 g of D_2O). To reach the final levels of metabolites concentrations in the tubes A, B, C, D and E (Figure 2), portions of the solution M1 prepared on big scale for the preparation of tube X were combined opportunely with the following two mixtures of metabolites in the buffer [$(HC_2O_4)^-/(C_2O_4)_2^-$ 0.11 mol L^{-1}, pH 4.2]: solution M2 was composed of glucose ($8.3\cdot10^{-1}$ mol L^{-1}) and arginine ($1.43\cdot10^{-3}$ mol L^{-1}); solution M3 was composed of fructose ($8.3\cdot10^{-2}$ mol L^{-1}) and alanine ($1.68\cdot10^{-4}$ mol L^{-1}). NMR tubes were filled with 0.700 mL of the resulting mixture, then flame-sealed and delivered to the laboratories.

Figure 2 here

Figure 2. The final five levels of metabolites concentrations reached in the spiked solutions which were contained in tubes A-E.

Data acquisition and processing. For each sample the participants were asked to perform five repetitions of a presaturated 1D ^1H NOESY NMR experiment,[29] optimized to remove the residual water signal with minimal affection of the baseline. The 5-fold replication was needed to comply with the conditions of intermediate precision, *i.e.* same NMR tube, same spectrometer, same user, at least 24 h delay between runs, removal of the NMR tube from the magnet from run to run. The participants received experimental instructions for setting the acquisition parameters according to the spectrometer manufacturer requirements.

For Varian/Agilent spectrometers, guidelines included: pulse program (NOESY); size of free induction decay (FID) (np, 128 K); spectral width (sw, 20 ppm); transmitter offset (tof): ca. 4.70 ppm (set the chemical shift value on the residual water signal); 90° hard pulse (pw, optimized by manual or automatic procedures keeping the pulse length as short as possible, preferably < 10 μs, if the hardware allows it); steady state (ss, 8); number of transients (nt, 64); mixing time (mixN, 0.010 s); recycle delay (d1, 5 s); no sspul (sspul = 'n'); no ZQ filter (Gzqfilt = 'n'); no homo spoil during mixing time (gt1 = 0, gzlvl1 = 0 and gstab = 0); presaturation during the whole length of d1, centered at the HDO residual signal with a nutation frequency of about 25 Hz [satmode = 'yn', satdly = d1, satfrq = tof; satpwr should be set to yield r1 of about 25 after running the command getpower(satpwr,tn):r1]; receiver gain optimization (once optimized for tube A, use the obtained receiver gain value also for all replicates and for all tubes A-E, X and Y).

For Bruker spectrometers, guidelines included: pulse program: noesypr1d; size of FID (TD, 128 K); spectral width (SW, 20 ppm); transmitter offset, ca. 4.70 ppm (set at the chemical shift value of the residual water signal); 90° hard pulse (p1, optimized by manual or automatic procedures keeping the pulse length as short as possible, preferably < 10 μs, if hardware allows it); power level for presaturation (pl9, calculated by command "pulse 25Hz" after optimization of p1); dummy scans (ds, 8); number of scans (ns, 64); mixing time (d8, 0.010 s); recycle delay (d1, 5 s); receiver gain optimization (once optimized for tube A, use the obtained receiver gain value also for all replicates and for all tubes A-E, X).

For JEOL spectrometers, guidelines included: pulse program: noesy_abs; y_points = 1; size of FID (x_point = 131072); spectral width (x_sweep = 20); transmitter offset (x_offset = 4.7); 90º hard pulse (x_pulse = x90; x_atn = xatn) to be optimized by manual or automatic procedures, keeping pulse length as short as possible, preferably < 10 μs; steady state (x_prescans = 8); number of transients (scans = 64); mixing time (mix_time = 0.010); recycle delay (relaxation_delay = 5 s); presaturation during the whole length of recycle delay, centered at the HDO residual signal with a γB_2 power of about 25 Hz (irr_mode = presaturation; irr-offset = x_offset; presat_time_flag = y); use the following formula to calculate the value of irr attenuator corresponding to 25 Hz: irr attenuation = x_atn + 20log(10.000/x90); receiver gain optimization (once optimized for tube A, use the obtained receiver gain value also for all replicates and for all tubes A-E, X).

The NMR raw data sets (FIDs and signal integrals) were uploaded by each laboratory on the website http://nmr.mxcs.it/index.php developed according to internationally agreed procedures.[30,31] The NMR spectra could be re-processed by Topspin 1.3 - AMIX 3.9.9 (Bruker BioSpin GmbH, Germany) and Mnova (Mestrelab Research, Spain) software. FIDs were extended by zero-filling to a final size of 256 K. Fourier transformation was performed by applying an exponential multiplication function with a line broadening factor of 0.1 Hz. Users could choose to apply either manual or automatic procedures for the phase and the baseline correction (without any limitations provided that the same

procedure be applied to all NMR spectra). The calculation of signal area was the only procedure accepted for signal integration. The TSP singlet signal (0.00 ppm) was used as internal reference compound, upon ascertaining it was stable in solution over time (see supplementary material for further details).

RESULTS AND DISCUSSION

Data analysis focused on the NMR signals related to alanine (S1), arginine (S2), glucose (S3, S6, and S7) and fructose (S4 and S5) as shown in figure 3. The signal intensities were calculated as the areas subtending the signals in the following regions: [1.42-1.51 ppm] for alanine (S1); [1.55-1.80 ppm] for arginine (S2); [3.19-3.29 ppm] for glucose (S3); [3.96-4.05 ppm] for fructose (S4); [4.06-4.15 ppm] for fructose (S5); [4.58-4.71 ppm] for glucose (S6); [5.15-5.31 ppm] for glucose (S7). Each signal intensity was scaled to that of TSP calculated in the range [–0.10-0.10 ppm]. The width of the integration intervals was opportunely chosen to overcome the small changes in the position and in the shape of the signals unavoidably occurring when the spectra are produced at different field strengths (see "Selection of spectra regions for signals integration" in supplementary material for further details). The interest towards alanine and arginine derives from the fact that these compounds are among the best yeast nitrogen sources during alcoholic and malolactic fermentation in wine production.[32] Moreover, their NMR signals offer the opportunity to evaluate the reproducibility of very weak signal intensities. Glucose and fructose were considered because of the importance of their quantification in wine grapes. Compared to other analytical techniques, NMR spectroscopy offers the advantage of identifying and quantifying glucose and fructose without previous separation. Five signals (S3, S6 and S7 belonging to glucose and S4 and S5 belonging to fructose) were considered not only with the aim of quantifying the two sugars but also to evaluate the critical issues deriving from proximity of the radiofrequency offset of the pre-saturation pulses to the signal frequencies.

Figure 3 here

Figure 3. Typical 1D ^{1}H-NOESY spectrum (700 MHz, D_2O) of NMR tube X containing an unspiked solution of grape juice (*cv*. Primitivo).

A total number of 65 regression lines, one per spectrometer, were developed for each selected signal by using the I_{signal}/I_{TSP} ratios (I_{signal} refers to the integral of the selected regions S1-S7 of the spectrum and I_{TSP} refers to the integral of the internal reference compound) calculated taking into account the five test tubes (A-E) containing the spiked juice solutions and the tube X containing the unspiked grape juice sample. Each tube was submitted to 5-fold replicated experiment. The 65 regression lines were subjected to five selection tests aimed to remove anomalous data and to select the suitable lines allowing for the development of the most performing regression models able to calculate the concentration of the selected metabolite. The first test (T1 – linearity) was applied to evaluate the linearity between the dependent variable *y* (I_{signal}/I_{TSP} values) and the independent variable *x* (spiked concentrations in tubes A-E and original concentration in tube X). Only the regression lines passing T1 by mean of the F-statistic, were admitted to the second test (T2 – slope distribution) to identify and reject the lines having slopes which were recognized as outliers by Huber and Cochran tests. In turn, the lines passing T2 were submitted to a similar procedure (T3 – *y*-intercept distribution) applied to the *y*-intercepts. All the variables belonging to the lines successfully passing T1, T2 and T3 were used to design a regression line which acted as a *community-built reference line* during the further two selection tests: T4 to select parallel lines (T4 – parallelism) and T5 to select coincident lines (T5 – coincidence).

As proof of concept, the following description refers to the selection process applied to S1 (alanine, 1.42-1.51 ppm). The results obtained from the study of the other signals are reported in the supplementary material. According to the procedure described above, the 65 regression lines were submitted to the selection tests T1-T5. The results are shown in Figure 4 where the red lines are those failing the selection tests and the grey lines are those successfully passing the tests.

Figure 4 here

Figure 4. Plots of the regression lines produced for the signal S1 during the sequence of selection tests: a. T1-linearity test; b. T2-slopes distribution (Huber and Cochran tests on slopes); c. T3-*y*-intercepts distribution (Huber and Cochran tests on *y*-intercepts); d. T4-parallelism test; e. T5-coincidence test. Grey lines passed successfully the selection tests; red lines failed the selection tests; black dashed line represents the community-built reference regression line.

T1 identified 2 lines (red lines in T1-linearity, Figure 4) which resulted not linear and, thus, were excluded during the subsequent steps. Considering the slopes of the remaining 63 regression lines, T2 identified 4 outliers (red lines in T2-Slopes distribution, Figure 4). The corresponding regression lines were removed from the data set. When the analogous test was applied to *y*-intercepts (T3), further 5 lines were rejected (red lines in T3-*y*-Intercepts distribution, Figure 4). The resulting set of calibration data was used to develop a new regression line which acted as a *community-built reference line* (Figure 4, dashed line) during the T4 and T5[33] to test the parallelism and the coincidence of each selected line with respect to the *community-built reference line* (see supplementary material for further details).

As a result of the described selection process, starting from initial 65 regression lines (ILC participants), only a reduced number of them passed successfully the sequence of T1-T5 tests, and could be deemed eligible for the quantification of the metabolites represented by the selected signals in the NMR spectrum (table 1).

Table 1. Number of regression lines produced by the 65 ILC participants for signals S1-S7 which succeeded the sequence of chemometric tests assessing their proficiency for quantification purposes.

	Test	S1	S2	S3	S4	S5	S6	S7
Entry 1	T1-Linearity	63	65	62	65	63	64	59
Entry 2	T2-Slope distribution	59	53	59	62	60	55	55
Entry 3	T3-*y*-Intercept distribution	54	49	58	59	56	54	54
Entry 4	T4-Parallelism	34	49	52	54	51	52	48
Entry 5	T5-Coincidence	25	20	17	22	18	9	14

As summarized in table 1, the number of lines with good level of linearity (59 to 65, table 1, entry 1) was high for all of the seven selected signals, confirming that the nuclei response is directly proportional to their concentration with an excellent linearity between the independent variable (metabolite concentration) and the dependent one (area under the signal). In addition, the number of lines remaining acceptable after the identification of outliers among the slopes and the *y*-intercepts (49 to 59, table 1, entry 3) was satisfactory. Such data further confirm the capability of the NMR spectroscopy to produce comparable lines, regardless the variability in the spectrometer features (see also supplementary material, tables S1-S7). As to the most demanding tests T4 and T5, T4 indicated that more than 50% of the lines (34 to 54, table 1, entry 4) could be safely considered parallel for all of the selected signals. T5 behaved noticeably as a bottleneck during the selection process for all the signals and caused a dramatic decrease in the number of regression lines which resulted coincident (9 to 25, table 1, entry 5) and thus, in principle, suitable for quantification. This behaviour was particularly pronounced for signal S6 and this can be ascribed to its proximity to the pre-saturation offset. Indeed, differences in the calibration of the power level used for pre-saturation, depending on both the hardware features and the skills of the operator, may enhance the random error associated with the intensity of the signals close to the pre-saturation frequency.

Since the T5 – coincidence test caused a dramatic reduction of the regression lines that, in principle, were suitable for quantification, it was evaluated if satisfactory results in terms of reproducibility of concentration values were conditional on passing the highly demanding T5 test. Accordingly, the reproducibility of the data obtained by the analysis of the parallel regression lines was compared with the one deriving from the analysis of coincident regression lines. In order to ascertain the suitability of the parallel lines passing T4 for a satisfactory quantification, the regression lines which resulted well-performing according to the test sequences T1-T4 and T1-T5 were employed to calculate the concentration of the metabolites represented by the signals S1-S7. The metabolite concentration in the tube X was estimated as $C = -x_{intercept}$, where $x_{intercept} = -b/a$ derived from the regression lines of general formula $y = ax + b$ after setting null values for *y*. The predicted values were then subjected to the Huber and Cochran tests for identification of possible outliers that were not considered in the next steps. The performance of the spectrometers was evaluated according to their z-score, as $z = (C_i - C_M)/\sigma$ where C_i represented the concentration predicted by each selected regression line, C_M represented the average predicted concentration by the set of all the selected regression models, and σ was the standard deviation calculated considering the corresponding predicted values (see supplementary material for further details).

Finally, the seven *community-built reference lines* were examined to get the values of the corresponding reference *x*-intercepts, which gave *predicted reference concentrations* (C_R) for the seven selected metabolites. Since C_R values were, in all cases, included in the "Horwitz region" ($1.2 \cdot 10^{-7} \leq C_R \leq 0.138$), for each signal the predicted relative standard deviation (PRSD%) was calculated by application of the Horwitz equation as $PRSD\% = 2^{(1-\log C_R)}$ (where C_R was expressed as mass/mass unit). The relative standard deviation (RSD%) of the predicted concentration was calculated as $RSD\% = \sigma/C_M \times 100$, where σ represented the standard deviation of the selected predicted values and C_M was the average of such values. The Horwitz ratio (HorRat), which is an accepted index assessing the data reproducibility in the context of interlaboratory comparisons, was evaluated as $HorRat = RSD\%/PRSD\%$. Such ratio, under reproducibility conditions, should be a value between 0.5 and 2.[34] As expected, the parallel regression curves gave RSD% values and, consequently, corresponding Horwitz ratios higher than those obtained from the coincident lines. Importantly, while the HorRat values for coincident lines ($HorRat_C$) were lower than 2 for all the seven signals, which could be defined as *statistically equivalent*, the HorRat values for parallel lines ($HorRat_P$) were higher than 2, and, thus, unsatisfactory for S2 and S6. As a result of this study, it was found that though the introduction of the coincidence test was important to obtain high reproducibility for all the selected metabolites independently of the considered signal, passing the T4 – parallelism test was sufficient to get satisfactory reproducibility for all of the signals but S2 and S6 (Table 2). It was ascertained (Tables S8, supplementary material) that such results were not related to spectrometer features such as manufacturer, magnetic field strength, hardware configuration, year of fabrication. Other factors might be considered as affecting the

instrumental response and thus the reproducibility. For instance, even though all the tubes were filled with a constant volume of the sample (0.700 mL, see experimental procedure), the instrumental response could differ depending on probe head features. Sample handling (delivering and storage) and the compliance of the local operative procedures with the guidelines might also affect the sample conditions and, consequently, NMR results. Nevertheless, based on the available data and information, no correlation of these factors with the observed results could be drawn.

Table 2. Average concentrations (C_M and C'_M) including the confidence interval predicted for the seven considered signals by the selected coincident (n_C) and parallel (n_P) regression curves, respectively. The reproducibility of the predicted average concentrations was evaluated by mean of the Horwitz ratios ($HorRat_C$ and $HorRat_P$ for the case of the coincident curves and the parallel ones, respectively). C_R (g/g), reference concentrations calculated according to the reference regression curve and included in the Horwitz equation. n, number of coincident and parallel regression curves, respectively, for each signal. PRSD, predicted relative standard deviation calculated according to the Horwitz equation. RSD_C and RSD_P, relative standard deviation of the metabolites concentration which were predicted by the coincident regression curves and by the parallel ones, respectively.

			T5 - Coincident lines				T4 - Parallel lines			
	C_R (g/g)	**PRSD (%)**	**n_C**	**C_M (g/g)**	**RSD_C (%)**	**$HorRat_C$**	**n_P**	**C'_M (g/g)**	**RSD_P (%)**	**$HorRat_P$**
S1 Alanine	2.25×10^{-5}	10.01	24	$2.22\times10^{-5}\pm0.15\times10^{-5}$	16.55	1.65	32	$2.25\times10^{-5}\pm0.14\times10^{-5}$	17.39	1.74
S2 Arginine	2.35×10^{-4}	7.04	15	$2.30\times10^{-4}\pm0.09\times10^{-4}$	8.14	1.16	45	$2.32\times10^{-4}\pm0.14\times10^{-4}$	20.88	2.97
S3 Glucose	4.84×10^{-2}	3.15	13	$4.91\times10^{-2}\pm0.04\times10^{-2}$	1.64	0.52	48	$4.86\times10^{-2}\pm0.04\times10^{-2}$	3.12	0.99
S4 Fructose	4.82×10^{-2}	3.16	22	$4.82\times10^{-2}\pm0.01\times10^{-2}$	5.3	1.68	54	$4.81\times10^{-2}\pm0.07\times10^{-2}$	5.17	1.63
S5 Fructose	5.18×10^{-2}	3.12	16	$5.16\times10^{-2}\pm0.11\times10^{-2}$	4.51	1.44	49	$5.09\times10^{-2}\pm0.08\times10^{-2}$	5.78	1.85
S6 Glucose	4.53×10^{-2}	3.19	8	$4.49\times10^{-2}\pm0.18\times10^{-2}$	5.77	1.81	52	$4.79\times10^{-2}\pm0.14\times10^{-2}$	10.56	3.31
S7 Glucose	4.75×10^{-2}	3.16	10	$4.74\times10^{-2}\pm0.08\times10^{-2}$	2.87	0.91	47	$4.94\times10^{-2}\pm0.08\times10^{-2}$	5.48	1.73

CONCLUSION

It has been explored the exclusive advantage offered by NMR spectroscopy to produce, for a given sample analysed by different spectrometers, a same instrumental response with high reproducibility. Such peculiarity, that is the independence of the instrumental response from magnetic field strength, hardware configuration, manufacturer and age, was opportunely exploited to design a pipeline on how to assess the reproducibility of quantitative NMR analysis. Results allowed to introduce a *community-built calibration system* able to perform quantitative analysis and to assess performance of the laboratories providing a reference calibration line along with a reference predicted concentration. The calibration data produced by 65 different spectrometers involved in an interlaboratory comparison were analysed and only the calibration lines passing T1-T3 (i.e. linearity, slope distribution and y-intercept distribution) tests were admitted to the development of the *community-built reference calibration* line. Therefore, the lines which resulted parallel and coincident with respect to the newly built reference line could be enrolled in the community-built calibration system, which was, ultimately, employed to quantify the analytes. The reproducibility of the predicted concentrations was highly satisfactory for all the selected metabolites, even when the set of parallel lines was the only one employed.

Many perspectives arise from the present study, paving the way for further development of this analytical approach, which ideally should give rise to a database comprising a number of community-built reference calibration lines for a range of analytes. Importantly, such database may be continuously updated based on the contribution of additional calibration lines which must be built under the same experimental conditions and, upon passing the described sequence of chemometric tests, must become suitable to be enrolled in the community-built calibration system. Consequently, the community-built reference line, taking advantage from the additional calibration data provided by the external laboratories should become progressively more accurate and precise, thus paving the way to an open interlaboratory comparison. An up-to-date calibration system may be advantageously used by the local laboratories to continuously evaluate their performance during the qNMR analysis of a given analyte.

This work may foster a more extensive use of qNMR method in standardized analytical protocols, overcoming the lack of official guidelines assessing the performance of qNMR analyses which still hampers the use of NMR methods as internationally accepted analytical protocols.

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