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1 Performance Assessment in Fingerprinting and Multi Component 2 Quantitative NMR Analyses

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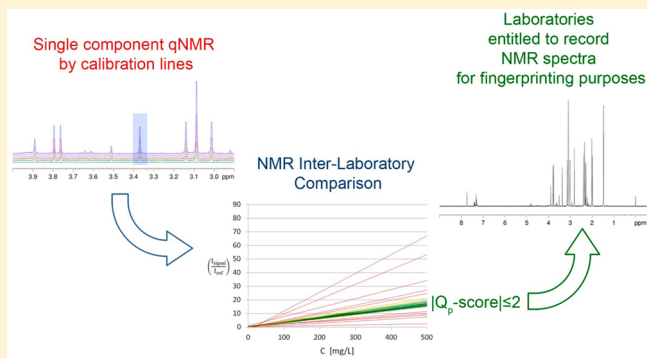
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16 **S** Supporting Information

17 **ABSTRACT:** An interlaboratory comparison (ILC) was
18 organized with the aim to set up quality control indicators
19 suitable for multicomponent quantitative analysis by nuclear
20 magnetic resonance (NMR) spectroscopy. A total of 36 NMR
21 data sets (corresponding to 1260 NMR spectra) were
22 produced by 30 participants using 34 NMR spectrometers.
23 The calibration line method was chosen for the quantification
24 of a five-component model mixture. Results show that
25 quantitative NMR is a robust quantification tool and that 26
26 out of 36 data sets resulted in statistically equivalent calibration
27 lines for all considered NMR signals. The performance of each
28 laboratory was assessed by means of a new performance index
29 (named Q_p -score) which is related to the difference between
30 the experimental and the consensus values of the slope of the
31 calibration lines. Laboratories endowed with a Q_p -score falling
32 within the suitable acceptability range are qualified to produce NMR spectra that can be considered statistically equivalent in
33 terms of relative intensities of the signals. In addition, the specific response of nuclei to the experimental excitation/relaxation
34 conditions was addressed by means of the parameter named NR. NR is related to the difference between the theoretical and the
consensus slopes of the calibration lines and is specific for each signal produced by a well-defined set of acquisition parameters.



35 **S**ince the first successful experiments on the detection of
36 nuclear resonance signals back in 1945–1946,^{1–3} nuclear
37 magnetic resonance (NMR) spectroscopy has become a
38 powerful technique for investigating the finer properties of
39 matter showing no sign of slackening even 70 years later. In the
40 field of quantitative analytical chemistry, the use of NMR as a
41 quantification tool has become very common for many
42 applications in both academic and industrial research such as
43 pharmacy, food, and materials science. Recently, the needs and
44 advantages of using NMR spectroscopy as a quantification tool
45 have been exhaustively reviewed by Bharti and Roy.⁴

46 NMR spectroscopy is considered a primary analytical
47 technique due to the possibility to derive a full uncertainty
48 budget by mathematical equations. As a consequence, NMR

spectroscopy is enabled for quantitative determinations at the
49 highest metrological level. The main feature making NMR a
50 powerful technique in quantitative determinations concerns the
51 direct proportionality existing between the intensity of the
52 NMR signal and the number of nuclei generating the signal.
53 Quantitative NMR does not need reference standard molecules
54 showing chemical structure similarity with the analyzed sample
55 as conversely requested, for instance, in chromatographic
56 methods. Quantification is typically obtained by integrating the
57

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58 signal of interest and scaling it to the peak area of a selected
59 signal generated by an arbitrary reference material, whose
60 concentration is known. Notwithstanding these advantages,
61 official qNMR methods are still rare,⁵ when compared to other
62 analytical techniques officially recognized for quantification.
63 The lack of official qNMR methods is a serious limitation for
64 the exploitation of NMR potential in single component
65 quantification analyses and represents a critical problem when
66 NMR potential is considered for multicomponent and
67 fingerprinting purposes. In fact, NMR spectroscopy is gaining
68 ever growing popularity for the development of analytical
69 approaches focusing on multicomponent untargeted anal-
70 yses.^{6–29} Among the many reasons for the gap between the
71 use of NMR and the use of other techniques for official
72 purposes, high costs of NMR spectrometers and high limits of
73 detection (LODs) are commonly invoked. However, the lack of
74 reproducibility data for specific methods also plays certainly an
75 important role in preventing recognition of NMR measure-
76 ments by institutions and certification bodies. This has to be
77 probably ascribed to the fact that academic researchers are
78 rarely involved in design of formal standardization procedures.
79 Measurement uncertainty is typically evaluated by three
80 models: one laboratory–one method (1L1M), many labo-
81 ratories–many methods (mLmM), and many laboratories–one
82 method (mL1M). In the NMR community, the first limit
83 model is the rule and several validation processes³⁰ are
84 available, demonstrating the suitability of NMR spectroscopy
85 as a quantification tool. For single component quantification,
86 the mLmM limit model is required for a wide acceptance of the
87 quantification method. Such a model was followed in the first
88 German and international interlaboratory comparisons organ-
89 ized by the Federal Institute of Materials Research and Testing
90 (BAM) in 1999.³¹ At that time, it was found that results
91 differed enormously (up to 100%) among the participating
92 laboratories. The unacceptable result was attributed to the
93 individual and independent setup of the measurements, the
94 data processing, and the evaluation procedure of each single
95 laboratory. To overcome these drawbacks, approximately 5
96 years later, another interlaboratory comparison was organized
97 by Melz and Jancke using the mL1M model for uncertainty
98 evaluation.³¹ The 33 participants used spectrometers working
99 at ¹H frequencies ranging from 200 to 600 MHz and adopted a
100 common protocol for the experimental setup and data
101 processing. The NMR experiment considered for this second
102 comparison consisted of a single 30° excitation pulse followed
103 by a suitable relaxation delay. Data elaboration, valid for
104 determination of mole ratios of each compound, turned out a
105 measurement uncertainty of 1.5% for a confidence level of 95%
106 ($k = 2$), thus demonstrating the importance of acquisition and
107 processing protocols for accurate and precise quantitative NMR
108 measurements. Moreover, it was demonstrated that precision
109 could be improved when a single operator processed all the
110 NMR spectra.

111 An interesting advantage of the NMR technique deals with
112 the possibility to suppress selectively one or more intense
113 signals with the consequent opportunity to enhance dramati-
114 cally the signal-to-noise ratio of weak signals. Typically, this
115 kind of experiment allows one to remove solvent signals thus
116 reducing the manipulation of the samples and avoiding the use
117 of large amounts of deuterated solvents. In routine experiments,
118 signal suppression can be simply obtained by implementing the
119 pulse sequence with a presaturation scheme consisting of a low

power radio frequency pulse able to saturate a specific
resonance.

In principle, the introduction of the presaturation scheme
should not affect the quantitative NMR measurements. The
reproducibility of a single pulse experiment preceded by
presaturation of the solvent signal has been evaluated by
application of principal component analysis (PCA) to ¹H NMR
data in the framework of two interlaboratory comparisons.^{32,33}
PCA offers the advantage to estimate measurement repro-
ducibility by easy visual inspection of the *scores* plot but quality
control indexes to be used as general reference parameters for
quality assessment of NMR spectra are still lacking.

With the aim to set up new quality control parameters
suitable for multi component quantitative NMR analysis as well
as for NMR fingerprinting methods, we have organized the first
Italian interlaboratory comparison according to the interna-
tionally agreed procedures ISO/IEC 17043:2010,³⁴ which
specifies general requirements for development and operation
of proficiency testing schemes, and ISO/IEC 17025:2005,³⁵
which specifies the general requirements for the competence to
carry out tests and calibrations performed using standard
methods, nonstandard methods, and laboratory-developed
methods. The conventional statistical elaboration of data was
carried out according to ISO 13528:2005³⁶ and ISO 5725, parts
1–6.³⁷ The analytical target of the comparison was the
quantification of analytes in a five-component model mixture
by the calibration curve approach and using the mL1M model
for uncertainty evaluation. Two different data elaborations were
considered: the first one was carried out by a single operator
who processed NMR spectra and developed calibration lines
with signal areas as input data, without referencing to any
standard molecule;³⁸ the second one was characterized by the
involvement of each participant in NMR spectra processing and
signal area calculation. In the second elaboration, signal areas
were scaled to a standard molecule and calibration lines were
developed by a specifically designed Web application.

In this paper, the comparison between results obtained by
the two data elaborations are discussed in terms of coefficient
of variation. The performance assessment in the second data
elaboration was carried out by means of the parameter (z -
score) usually considered as performance index in single
component quantifications as well as by means of a new
parameter, named Q_p -score, better suited for performance
assessment in multicomponent and fingerprinting analyses.
Moreover, a third index (NR), specific for each NMR signal,
was introduced to gain insights into the possible effects of the
acquisition parameters on signal intensities.

EXPERIMENTAL SECTION

Materials. 2-Methyl-2-(methylthio)propanal-*O*-(*N*-
methylcarbamoyl)oxime (Aldicarb, CAS No. 116-06-3, neat
purity 99.9%, Sigma-Aldrich, Milan, Italy), 2-methoxy-*N*-(2-
oxo-1,3-oxazolidin-3-yl)-acet-2',6'-xylylide (Oxadixyl, CAS No.
77732-09-3, neat purity 99.9%, Sigma-Aldrich, Milan, Italy),
O,S-dimethylphosphoramidothioate (Methamidophos, CAS
No. 102658-92-6, neat purity 98.5%, Sigma-Aldrich, Milan,
Italy), (2-dimethylamino-5,6-dimethylpyrimidin-4-yl)-*N,N*-di-
methylcarbamate (Pirimicarb, CAS No. 23103-98-2, neat purity
99.0%, Sigma-Aldrich, Milan, Italy), 3-(trimethylsilyl)-2,2,3,3-
tetra-deutero-propionic acid sodium salt (TSP, CAS No. 24493-
21-8, 99% D, Armar Chemicals, Döttingen, Switzerland),
deuterium oxide (D₂O, CAS No. 7789-20-0, 99.86% D,
Sigma-Aldrich, Milan, Italy) were used for sample preparation.

182 Chemical structures of compounds are reported in Chart S1 in
183 the Supporting Information.

184 **Sample Preparation.** Standard and test mixtures were
185 prepared under thermic and hygrometric control (20 ± 5 °C,
186 40–60 R.H.%) by gravimetric method using a certified
187 analytical balance KERN ABT 100-5 M (KERN & Sohn
188 GmbH, Balingen, Germany) with weighing range 1–101.000
189 mg, readability 0.01 mg, and reproducibility 0.05 mg. The
190 balance was periodically calibrated by the certified test weight
191 set KERN DKD-K-11801, 11-06, s/n G0703552. Uncertainty
192 for each analyte mass was calculated taking into account
193 uncertainty parameters of the balance. A factor $k = 2$,
194 corresponding to a confidence level of 95%, was considered
195 to determine extended uncertainties.

196 A solution made up of TSP in D₂O (20.33 ± 0.29 mg/L) was
197 used to prepare six standard (labeled as A–E and Blank) and
198 one test (labeled as X) mixtures at the levels listed in Table S1
199 in the Supporting Information (in the range 0–500 mg/L).
200 Standard mixtures were used to obtain the calibration curves
201 considered to determine the concentration values of the test
202 mixture X. Mixtures were prepared by diluting stock solutions
203 to the desired concentration using class A glassware. NMR
204 tubes were filled in with 1.0 mL of the solution.

205 **Experimental Procedures.** Nine NMR signals were
206 selected for this study: three for Aldicarb (A1, A2 and A3),
207 one for Methamidophos (M1), two for Oxadixyl (O1 and O2),
208 two for Pirimicarb (P1 and P2), and the singlet of TSP which
209 was taken as reference. A typical ¹H NMR spectrum of the
210 mixture is reported in Figure S1 in the Supporting Information
211 and the integration ranges used for calculation of the peak areas
212 are listed in Table S2 in the Supporting Information.

213 In order to choose the optimal recycle delay, T₁ values were
214 determined taking into proper account all signals listed in Table
215 S2 in the Supporting Information. T₁ determination was carried
216 out by inversion recovery experiments applied to single
217 component solutions (analyte in D₂O) at two different
218 magnetic fields, 9.4 T (400 MHz) and 16.5 T (700 MHz),
219 and two concentration levels, ~37 mg/L and ~600 mg/L. The
220 highest T₁ value (5.4 s, measured for M1 signal of a 37.4 mg/L
221 solution of Methamidophos at 9.4 T) was taken into account to
222 set the recycle delay to 30 s. D₂O was not degassed before
223 preparation of the solutions. Single component solutions and
224 test mixtures were prepared in the same laboratory using the
225 same batch of D₂O. NMR tubes were filled with 0.5 mL
226 solution, sealed, and delivered to the participants.

227 **Data Acquisition and Processing.** The NMR experiment
228 considered for the interlaboratory comparison consisted of a
229 single 90° excitation pulse preceded by a selective presaturation
230 step. Even though it was organized before the publication of the
231 EUROLAB technical report on NMR method development
232 and validation,³⁹ this work produced results coherent with
233 guidelines described therein. For each NMR tube, 5 spectra
234 were recorded to comply with conditions for repeatability
235 (measurements performed under the same operating condi-
236 tions over a short period of time) considering the same NMR
237 tube, same spectrometer, same user, consecutive runs without
238 removing the NMR tube from the magnet and to comply with
239 conditions for intermediate precision (measurements per-
240 formed under repeatability condition devoid of only one
241 obligation) considering the same NMR tube, same spectrom-
242 eter, same user, at least 24 h delay between runs, removal of the
243 NMR tube from the magnet from run to run. Summarizing,
244 each participant recorded 35 NMR spectra (5 replicates for

each of the 7 NMR tubes) in three different sessions: (i) 3
consecutive runs per NMR tube (run 1, run 2, and run 3); (ii) 1
run per NMR tube delayed at least 24 h from the first session
(run 4); (iii) 1 run per NMR tube delayed at least 24 h from
the second session (run 5). It has been demonstrated³⁸ that
results obtained in repeatability conditions (considering only
data obtained by runs 1–3), in intermediate precision
conditions (considering only data obtained by runs 1, 4, and
5) and both conditions (considering data obtained by runs 1–
5) can be safely considered as substantially equivalent. In the
present paper, calculation on all available replicates (runs 1–5)
will be described. More details on NMR data acquisition and
processing are reported in the Supporting Information.

Statistical Elaboration. Signal integrals were scaled to the
TSP integral and the corresponding ($I_{\text{signal}}/I_{\text{TSP}}$) values were
uploaded on a Web application specifically designed and
validated for data elaboration in agreement with internationally
accepted requirements.^{35–37} ($I_{\text{signal}}/I_{\text{TSP}}$) values were uploaded
reporting at least four decimal places. The five ($I_{\text{signal}}/I_{\text{TSP}}$)
replicates collected for each signal and for each NMR tube were
submitted to the Shapiro-Wilk test to ascertain their normal
distribution and to Huber, Dixon, and Grubbs tests for
identification of possible outliers. Throughout the paper,
Grubbs tests refer to application of both the classical Grubbs
test identifying one outlier and the double Grubbs test which
enables the identification of two outliers. Data identified as
outliers by all the four tests were not considered in successive
steps. Data derived from standard mixtures A–E and Blank
were used to plot ($I_{\text{signal}}/I_{\text{TSP}}$) versus analyte concentrations
and to develop an equation for the calibration line by least-
squares linear regression. The equation of general formula $y =$
 $ax + b$ (with $y = (I_{\text{signal}}/I_{\text{TSP}})$ and $x =$ concentration as mg/L)
was used to calculate concentration values of analytes in test
mixture X. Then, the 5 concentration values calculated for the
test mixture X were submitted to the Shapiro-Wilk test to
ascertain their normal distribution and to Huber, Dixon, and
Grubbs tests for identification of possible outliers. After
removing outliers, calculated concentrations were used to
determine the mean concentration values and the correspond-
ing standard deviations which were considered as intra-
laboratory uncertainties of the method. Results from all
participants (36 sets of results from 34 NMR spectrometers)
were submitted to data elaboration for proficiency test and for
determination of the assigned values for analytes in mixture X.
The lack of official qNMR analyses for this case study
prompted us to determine assigned values as consensus values
from participants.³⁴ Thus, for each analyte, according to the
flowchart suggested by Horwitz,⁴⁰ the 36 standard deviation
values were submitted to the Cochran test (provided that all of
the 5 replicates of mixture X successfully passed the above-
mentioned tests for outliers) with the aim to identify and
remove outliers for successive calculations. In turn, mean
concentration values from data sets which passed successfully
the Cochran test were submitted to Grubbs tests with the aim
to further refine the quality of the results. The remaining sets of
data were submitted to the Shapiro-Wilk test to ascertain the
normal distribution of the population (data were always normal
distributed after refinement by the Cochran and Grubbs tests)
and were used to calculate, for each analyte in test mixture X,
the assigned concentration value, the interlaboratory standard
deviation, the coefficient of variation (CV%), and the
reproducibility limits.

RESULTS AND DISCUSSION

Performance Assessment for Single Component Quantitative NMR Measurements. Among the quantification approaches available for NMR spectroscopy,⁴ the calibration line method was chosen in this work as it allows for identification of a theoretical line to be taken as reference in performance assessment. Moreover, this method has a general applicability in analytical chemistry and has the advantage to nullify the effects of nuclei relaxation on quantitative accuracy, provided that all the acquisition parameters are kept constant for standard and test solutions.⁴ Thus, it is expected that systematic errors deriving from hardware features or from the set of acquisition parameters should be minimized.

A first statistical data elaboration of the ILC was carried out by a single operator who processed NMR spectra (Fourier transformation, phase and baseline correction, signal integration) and obtained calibration lines with no scaled signal areas as input data.³⁸ In a second data elaboration, NMR data processing was carried out by each participant and signal areas were scaled to the TSP area. Therefore, the main difference between the two elaboration approaches relays on different processing conditions. Results of both elaborations are summarized in Table S3 in the Supporting Information where assigned concentration values along with the corresponding standard deviations, coefficients of variation, and reproducibility limits are reported. It is apparent that changing the processing conditions of the NMR spectra, from “one operator—all NMR data sets” to “one operator—one NMR data set”, has a little impact on the final result in terms of mean value. Conversely, standard deviations (and consequently the related coefficients of variation and reproducibility limits) are affected by the different NMR processing conditions. Notwithstanding the deterioration of their quality in terms of coefficient of variation (CV%), these results are quite satisfactory if this test is considered as a confirmatory method for organic residues and contaminants. Indeed, according to the European Commission decision concerning the performance of analytical methods and the interpretation of results,⁴¹ the interlaboratory coefficient of variation (CV%) for repeated analysis of a reference material, under reproducibility conditions, shall not exceed 5.7% for concentration values higher than 1000 ppm, according to the Horwitz equation:

$$CV\% = 2^{(1-0.5 \log C)}$$

where C is the mass fraction expressed as a power of 10 (e.g., 1 mg/g = 10^{-3}). Being the concentration values considered in this work are lower than 500 mg/L, the highest obtained CV% value of 4.9% indicates that single excitation pulse preceded by selective presaturation of the solvent is a reliable NMR experiment for quantification purposes.

Once the assigned values for all the analytes were determined, performance statistics were carried out with the aim to estimate the deviation of the mean concentration values from the assigned value for each participant, including those producing data sets rejected by the Cochran and Grubbs tests. A commonly used parameter estimating the performance for quantitative results is the z -score, which is defined as

$$z = \frac{C_i - \bar{C}}{\sigma}$$

where C_i is the mean concentration value determined by the i th data set, \bar{C} is the assigned concentration value, and σ is the

interlaboratory standard error, all referred to as a single NMR signal. Satisfactory performance is indicated by $|z| \leq 2.0$, questionable performance is obtained when $2.0 < |z| < 3.0$, while $|z| \geq 3.0$ indicates unsatisfactory performance. In the latter case, suitable actions are required to identify and to solve the analytical problems.

Figure 1 shows the z -scores of Aldicarb quantification by the NMR A1 singlet. It is apparent that, even though results of 10

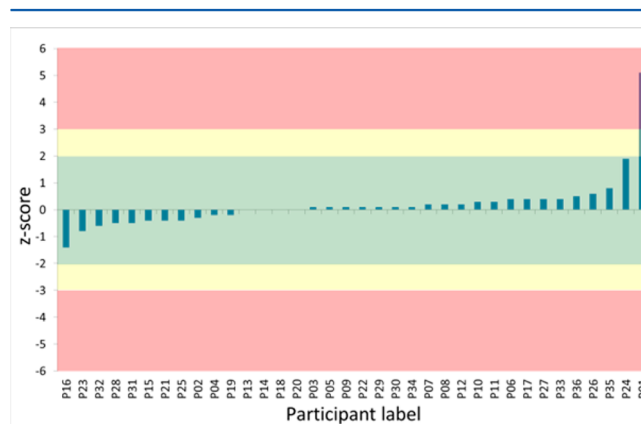


Figure 1. z -score for quantification of Aldicarb by means of A1 signal (green, $|z| \leq 2.0$; yellow, $2.0 < |z| < 3.0$; red, $|z| \geq 3.0$). Assigned concentration value, 94.57 mg/L; interlaboratory standard deviation, 3.64; reproducibility limit, 12.46; CV%, 3.8%).

participants were excluded from calculation of the assigned value, the quality of the result was satisfactory for 35 sets of NMR data and only 1 unsatisfactory performance was registered. Very similar results were obtained using each of all other NMR signals (Supporting Information, Figures S2–S8). High-performance quantifications are obtained also when signals different from singlets were taken into account (as in the case of M1 and O2 where a doublet and a group of signals were considered, respectively). It is worth noting that performance in terms of result quality was not affected by the magnetic field, hardware configuration, manufacturer, and production year of the spectrometer. These findings highlight the robustness of NMR spectroscopy when calibration curve approaches are adopted.

The z -score represents a satisfactory indicator for performance assessment in single component analyses, but it cannot account for performance assessment in multicomponent analyses because a single z -score refers to only a single quantification measurement. Thus, for performance assessment in fingerprinting measurements and quantitative multicomponent analyses, the introduction of indicators more appropriate than the z -score is desirable.

Basics of Quantitative NMR. Before discussing the new quality control parameters proposed by us in performance assessment for fingerprinting measurements and quantitative multicomponent analyses, recall of the basic equation of quantitative NMR is advisable (eq 1).

$$I = kn \quad (1)$$

Equation 1 provides the direct proportionality between the number of moles (n) of nuclei generating a signal and the intensity (I) of the same signal with a proportionality constant k being the spectrometer constant which remains the same for all resonances in a NMR spectrum.⁴

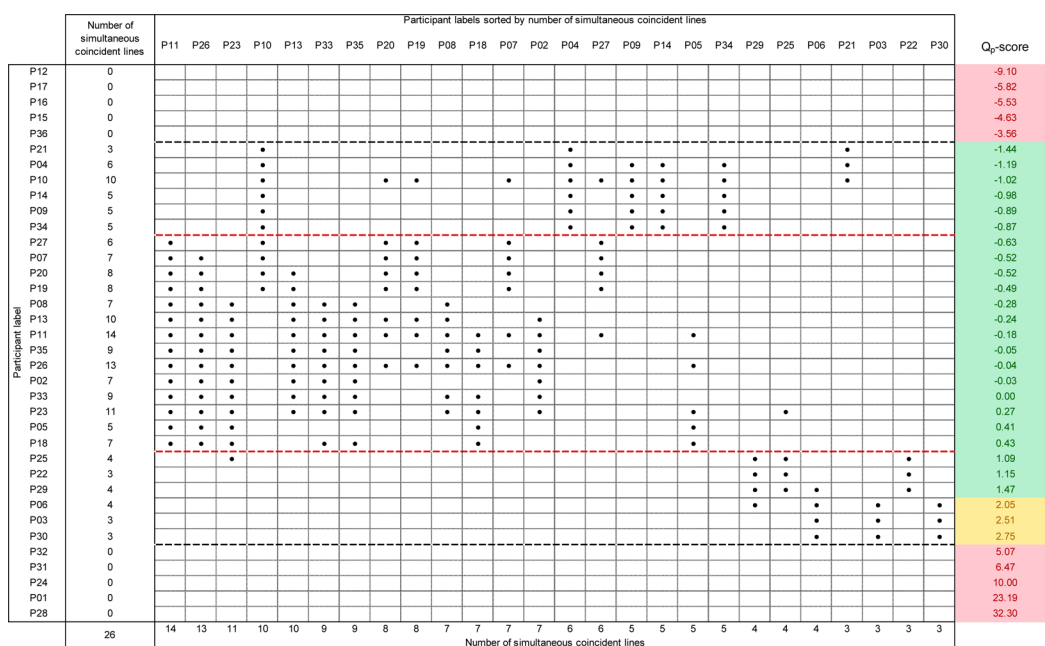


Figure 2. Results of the paired t test for statistical equivalence of pairs of calibration lines and laboratory Q_p -scores (referred to the A1 signal). Equivalent lines are cross-linked by the ●; green, $|Q_p| \leq 2.0$; yellow, $2.0 < |Q_p| < 3.0$; red, $|Q_p| \geq 3.0$.

Let us consider, in a NMR spectrum, the signal (a) having intensity I_a generated by specific protons belonging to the analyte of interest and the signal (r) having intensity I_r generated by specific protons in a reference compound. Applying eq 1 to I_a and I_r gives

$$I_a = kn_a$$

$$I_r = kn_r$$

Hence the ratio $(I_a/I_r) = (n_a/n_r)$ (eq 2) is independent from the proportionality constant k and, as a consequence, it does not depend on the spectrometer. Thus, taking the methyl protons signal of TSP as reference signal, all of the calibration lines obtained plotting (I_a/I_{TSP}) versus analyte concentration (C) should be independent from the spectrometer and statistically equivalent to each other. In other words, all the participants to an ILC should develop equivalent calibration lines

$$\left(\frac{I_a}{I_{TSP}}\right) = aC + b \quad (3)$$

where intercept b should have a null value due to the fact that no signal is generated if no nuclei ($C = 0$ mg/L) are contained in the mixture. Thus, eq 3 can be rewritten as

$$\left(\frac{I_a}{I_{TSP}}\right) = \left(\frac{n_a}{n_{TSP}}\right) = \left(\frac{\frac{m_{\text{analyte}}}{M_{\text{analyte}}} N_a}{\frac{m_{TSP}}{M_{TSP}} N_{TSP}}\right) = aC = a \frac{m_{\text{analyte}}}{V} \quad (4)$$

where m_{analyte} is the mass of the analyte, m_{TSP} is the mass of TSP, M_{analyte} is the molar mass of the analyte, M_{TSP} is the molar mass of TSP, N_a is the number of protons generating the signal (a), N_{TSP} is the number of methyl protons (nine) generating the reference signal, and V the solution volume.

Equation 4 can be rearranged into

$$\left(\frac{\frac{m_{\text{analyte}}}{M_{\text{analyte}}} N_a}{\frac{m_{TSP}}{M_{TSP}} N_{TSP}}\right) = a \frac{m_{\text{analyte}}}{V} \quad (5)$$

From eq 5 the theoretical value that slope must assume for a given TSP concentration can be extracted:

$$a_{\text{theoretical}} = \frac{M_{TSP}}{M_{\text{analyte}}} \frac{N_a}{N_{TSP}} \frac{V}{m_{TSP}} = \frac{M_{TSP}}{M_{\text{analyte}}} \frac{N_a}{9} \frac{1}{C_{TSP}} \quad (6)$$

The need to harmonize NMR protocols prompted us to propose a new parameter suited for checking the equivalence of the calibration lines. Such a parameter will be shown to represent a quality control index of the NMR spectra to use in fingerprinting applications and multicomponent NMR quantifications.

Quality Control Parameters for Performance Assessment in Fingerprinting Measurements and Quantitative Multicomponent Analyses. In order to assess the laboratory performance in multicomponent analyses without considering as many z -scores as the number of analytes, we propose a new parameter, named Q_p -score, accounting for participant performance as the result of instrumental adequacy and operator skill. Knowing that, for each signal, calibration lines developed by each participant must be equivalent to each other, let us define the indicator of the line equivalence Q_p as

$$Q_p = \frac{a_i - \bar{a}}{\sigma_{\text{slope}}} \quad (7)$$

where a_i is the slope of the calibration line determined by the i th participant, \bar{a} is the consensus slope value, and σ_{slope} is the interlaboratory standard deviation on slopes, all referred to a single NMR signal. The values \bar{a} and σ_{slope} are determined using a_i successfully passing the Huber test. By an analogous reasoning followed for the z -score, performance assessment by the Q_p -score is considered satisfactory when $|Q_p| \leq 2.0$, 456

Participant label	Signal							
	A1	A2	A3	M1	O1	O2	P1	P2
P12	-9.10	-8.07	-6.46	-6.62	-8.55	-6.67	-6.59	-6.68
P17	-5.82	-5.29	-4.03	-4.21	-5.50	-4.29	-4.29	-4.19
P16	-5.53	-4.90	-4.08	-4.07	-5.35	-4.18	-4.01	-4.00
P15	-4.63	-4.12	-3.25	-3.34	-4.33	-3.33	-3.31	-3.32
P36	-3.56	-3.20	-2.67	-2.60	-3.39	-2.83	-2.56	-2.58
P21	-1.44	-1.34	-1.11	0.22	-0.68	0.78	-1.21	-1.01
P04	-1.19	-1.11	-0.84	-0.82	-1.20	-0.96	-0.82	-0.82
P10	-1.02	-0.99	-0.71	-0.71	-1.04	-0.78	-0.72	-0.68
P14	-0.98	-1.00	-0.96	-0.86	-0.89	-0.66	-1.05	-1.03
P09	-0.89	-0.96	-0.84	-0.71	-0.86	-0.73	-0.75	-0.74
P34	-0.87	-0.91	-0.77	-0.58	-1.04	-0.75	-0.49	-0.83
P27	-0.63	-0.62	-0.42	-0.36	-0.71	-0.47	-0.29	-0.60
P07	-0.52	-0.65	-0.39	-0.29	-0.48	-0.39	-0.35	-0.31
P20	-0.52	-0.50	-0.37	-0.48	-0.59	-0.46	-0.30	-0.25
P19	-0.49	-0.55	-0.32	-0.29	-0.67	-0.39	-0.29	-0.14
P08	-0.28	-0.39	-0.20	-0.06	-0.34	-0.23	-0.18	-0.01
P13	-0.24	-0.37	-0.13	-0.07	-0.25	-0.19	-0.14	0.01
P11	-0.18	-0.22	1.03	-0.72	-1.13	-0.90	-0.46	-0.82
P35	-0.05	-0.26	0.21	-0.04	-0.03	-0.20	-0.10	0.08
P26	-0.04	-0.23	-0.10	-1.28	-0.06	0.41	-0.38	-0.25
P02	-0.03	-0.15	-0.31	0.13	0.16	-0.45	0.10	0.01
P33	0.00	0.06	-0.22	-0.03	0.15	-0.05	0.14	-0.01
P23	0.27	0.54	0.65	0.11	-0.07	0.86	0.50	0.59
P05	0.41	0.22	0.33	0.44	0.17	0.21	0.32	0.54
P18	0.43	0.40	0.22	0.49	0.50	0.44	0.47	0.62
P25	1.09	0.80	0.77	0.83	0.75	0.71	0.86	1.04
P22	1.15	0.80	0.77	0.99	0.83	0.84	1.02	0.80
P29	1.47	1.20	1.08	1.14	1.16	0.97	1.12	1.01
P06	2.05	1.86	1.63	1.56	1.80	1.53	1.59	1.53
P03	2.51	2.08	1.72	1.85	2.15	1.68	1.90	1.74
P30	2.75	2.32	1.96	2.13	2.38	1.99	2.08	2.11
P32	5.07	4.28	3.56	3.83	4.49	3.68	3.82	3.73
P31	6.47	5.54	4.62	4.91	5.82	4.77	4.88	4.76
P24	10.00	8.62	7.08	7.52	9.27	7.27	7.50	7.43
P01	23.19	20.03	16.33	15.56	20.26	16.45	16.51	16.56
P28	32.30	28.19	22.62	22.61	28.75	23.11	23.21	23.35

Figure 3. Q_p -scores for all NMR signals as labeled in Table S2 in the Supporting Information. Green, $|Q_p| \leq 2.0$; yellow, $2.0 < |Q_p| < 3.0$; red, $|Q_p| \geq 3.0$.

questionable when $2.0 < |Q_p| < 3.0$ and unsatisfactory when $|Q_p| \geq 3.0$.

In the case study of Aldicarb quantification by NMR signal A1, Huber tests applied to the 36 slope values gave 11 outliers, the 5 lowest and the 6 highest values. The 25 remaining values resulted in a normal distribution after the Shapiro-Wilk test and were considered for the calculation of \bar{a} and σ_{slope} . The values of \bar{a} and σ_{slope} were 0.0340 L/mg and 0.0032 L/mg, respectively.

Concerning the experimental intercept values, the population was not normal and was too scattered so that iterated Huber test gave meaningless results (all values were identified as outliers). The mean value of the intercept was -0.048 , and the related standard deviation was 0.513 indicating that the null value can be well considered as the experimental intercept.

These results give $y = 0.0340x$ as the consensus equation for the calibration line but do not yet demonstrate the statistical equivalence of the calibration lines. In order to evaluate the statistical parallelism, and then the equivalence of the calibration lines, all possible slope pairs were submitted to the paired t test with 95% confidence level. Computational part of the test consists of calculation of parameter t_{slopes} as the difference between two slopes divided by the standard error of the difference between the same two slopes.⁴² Then, t_{slopes} was compared with Student's t at the desired confidence level (95%) to evaluate whether the null hypothesis was supported, that is whether no relationship between two data sets existed. If the slope obtained by one data population is significantly different from that generated with another (and independent) data set, then $t_{\text{slopes}} > t$ or else $t_{\text{slopes}} \leq t$, and the two slopes can be considered statistically equivalent. Results of the paired t tests applied to all possible slope pairs are summarized in Figure 2 where statistically equivalent lines are cross-linked by black circles. For instance, participant P11 produced a calibration line which is equivalent to those produced by

participants P27, P07, P20, P19, P08, P13, P35, P26, P02, P33, P23, P05, and P18.

It is apparent from Figure 2 that the slopes of 26 calibration lines (bordered by black dashed lines) are statistically equivalent. Of these 26 lines, 23 were characterized by $|Q_p|$ lower than 2, i.e., in the range of satisfactory performance assessment. Moreover, with the exception of participant P10, the highest number of simultaneous coincidences (7–14) was recorded for participants endowed with very low Q_p -scores (ranging from -0.63 to 0.43 , bordered by red dashed lines in Figure 2).

The statistical equivalence of the calibration lines is in agreement with the theoretical treatment described above. It represents the experimental evidence that, for a given TSP concentration, slopes assume a certain value depending on the signal and not on the spectrometer constants. Once defined, the concentration range of the analytes, slopes associated with satisfactory Q_p -scores indicate that the corresponding NMR spectra were recorded and processed under similar conditions. Deviation from the consensus value of the slope is explained in terms of hardware reliability, acquisition and processing parameters. Therefore, Q_p -score represents a quality control index which accounts for hardware functioning conditions and operator skills. It is important to point out that questionable and unsatisfactory Q_p -scores did not prevent successful single component quantifications as the latter depend only on the quality of the calibration line. Indeed, good fitting in the linear regression allows for a good performance in terms of z -score but it does not account for deviation of the slope from its theoretical value.

Given that the Q_p -score is a quality parameter of the NMR spectrum as a whole, it can be expected that, as far as multicomponent analysis is concerned, Q_p -score based performance assessment of a laboratory should be almost independent from the considered signal. This is indeed the case, as clearly

Table 1. NR Values (%) Calculated for All NMR Signals As Labeled in Table S2 in the Supporting Information^a

signal	A1	A2	A3	M1	O1	O2	P1	P2
M_{analyte} (g/mol)	190	190	190	141	278	278	238	238
N_a	6	3	1	3	3	3	3	3
$a_{\text{theoretical}} \times 10^2$ (L/mg)	2.97	1.48	0.49	2.00	1.01	1.01	1.19	1.19
$\bar{a} \times 10^2$ (L/mg)	3.40	1.72	0.54	1.95	0.95	1.20	1.39	1.38
NR (%)	-14.6	-15.7	-9.3	2.4	6.2	-18.2	-17.0	-16.7

^a $M_{\text{TSP}} = 172.27$ g/mol; $N_{\text{TSP}} = 9$; $C_{\text{TSP}} = 20.33$ mg/L.

demonstrated by inspection of Figure 3, where Q_p -scores obtained by each participant by considering each of the NMR signals selected for this study are reported. Apart from the variations of the Q_p -scores falling in the proximity of the limiting value ± 2 , the performance category ($|Q_p| \leq 2.0$, $2.0 < |Q_p| < 3.0$ and $|Q_p| \geq 3.0$) is retained for all considered signals. These findings are in good agreement with the high reproducibility of ^1H NMR experiments evaluated by PCA in previous studies.^{32,33}

The introduction of Q_p -score paves the way to validation of multicomponent quantification methods, of great importance for fingerprinting and profiling applications. In fact, such validation procedures might be carried out in the future by an interlaboratory comparison where laboratory performance could be preliminarily assessed developing calibration lines for any arbitrary compound mixture. Once a Q_p acceptability range is fixed (for instance, $|Q_p| < 1$), all laboratories within such a range will be qualified to produce NMR spectra of a given mixture that are statistically equivalent in terms of relative intensities of the signals. In other words, for a given set of acquisition parameters, laboratories gaining satisfactory Q_p -scores will be accredited to record NMR spectra on every kind of mixture, thus allowing for pooling of NMR data in suitable databanks.

It is worth noting that, in real experiments, a deviation from theoretical slope is expected due to the specific response of the nuclei to the experienced excitation/relaxation conditions during spectrum acquisition. Such a response depends on several factors including (i) hard excitation pulse which must be uniform throughout all the spectral width; (ii) proximity of the signals to the offsets; (iii) recycle delay, which must be long enough to allow for complete magnetization recovery of all nuclei; (iv) energy exchange effects (NOE, spin diffusion, etc.) introduced by soft pulses. Therefore, in any interlaboratory comparison the consensus slope may differ from the theoretical one as an effect of the specific set of acquisition parameters.

In order to gain insights into the effects of the experimental excitation/relaxation conditions on the nuclei response, we introduce a new indicator as the relative deviation of the consensus slope from the theoretical value, according to eq 8.

$$\text{NR} = \frac{a_{\text{theoretical}} - \bar{a}}{a_{\text{theoretical}}} \times 100 \quad (8)$$

NR calculated for all signals considered in this study are reported in Table 1.

NR values for the various signals ranged from -18.2% to 6.2% indicating that signals are not affected to the same extent by the used acquisition parameters. Moreover, NR values were different also for signals generated by inequivalent nuclei in the same molecule.

In the present case, NR represents an index of the response of the nuclei submitted to a NMR experiment characterized by a single 90° excitation pulse preceded by a selective

presaturation step with the specific set of acquisition parameters. In our opinion, among the above-mentioned factors affecting the nuclei response, energy exchange effects introduced by soft pulses can be considered the most relevant to interpret the NR values obtained in the present study. Energy exchange effects are certainly operative in the acquisition condition characterized by a selective pulse acting during the long recycle delay (30 s). The other factors are thought to affect NR values only marginally because possible incorrect setting of the pulses (factors i and ii) will give random contributions averaged to almost null deviation of the calibration line and because the adopted recycle delay (longer than 5 times the highest measured T_1) ensures complete recovery of the magnetization (factor iii). Anyway, a deeper study on factors affecting the nuclei response to experimental acquisition conditions to give the NR values reported in Table 1 requires further NMR experiments. This is out of the scope of the present paper.

CONCLUSION

This study introduces a new quality control parameter, Q_p -score, suitable for harmonization of fingerprinting protocols and quantitative multicomponent analysis. Such a parameter, that was designed considering consolidated internationally agreed statistics, represents an unbiased evaluation tools for NMR method validations.

The Q_p -score accounts for laboratory performance in terms of both instrumental adequacy and operator skill and enables laboratories to pooling of NMR data in suitable databanks. Moreover, Q_p can be valuable for the development of multilaboratory metabolomic platforms. In fact, it was shown that participants having a Q_p -score in a suitable acceptability range are able to produce NMR spectra of a given mixture that can be considered statistically equivalent in terms of relative intensities of the signals. Another practical use of Q_p -score consists of the entitlement of laboratories endowed with acceptable $|Q_p|$ values to carry out quantifications by using relative intensity of the signal of interest after fitting with the consensus calibration line deriving from the interlaboratory comparison. For instance, in suitable networking conditions, equivalent calibration lines could be shared to enable different laboratories to carry out quantitative analyses without wasting time in calibration steps, with a consequent increase of productivity.

Since basic equations of quantitative NMR are independent from the type of solvent, it can be expected that performance assessment by Q_p -score applies also to experiments carried out in solvents others than D_2O (for instance, in organic solvents such as CDCl_3 widely used in lipidomics and in complex mixtures such as biofluids which are mostly used in their native state).

Another parameter, NR, has been proposed, which is related to differences between the theoretical and the consensus slopes

629 of the calibration lines and which is specific for each signal
630 produced by a well-defined set of acquisition parameters. For a
631 given molecule in a defined solvent, NR represents an index of
632 the specific response of the various nuclei submitted to a
633 definite NMR experiment.

634 ■ ASSOCIATED CONTENT

635 ● Supporting Information

636 Complete list of affiliations and complete list of acknowledged
637 individuals; chart of chemical structures of five components
638 contained in the model mixture; Tables S1 (analyte
639 concentration values assessed by gravimetric method in
640 standard and test mixtures), S2 (signal labels, chemical shifts
641 and integration ranges used for the calculation of peak areas),
642 and S3 (comparison between two data elaboration ap-
643 proaches); data acquisition protocol; NMR data processing;
644 and a typical ¹H NMR spectra of the mixture (Figure S1) and
645 additional z-score plots (Figures S2–S8). The Supporting
646 Information is available free of charge on the ACS Publications
647 website at DOI: 10.1021/acs.analchem.5b00919.

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653 The manuscript was written through contributions of all
654 authors. All authors have given approval to the final version of
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656 Notes

657 The authors declare no competing financial interest.
658 ^{4–33}The complete list of the affiliations is reported in the
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