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Performance Assessment in Fingerprinting and Multi Component Quantitative NMR Analyses

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

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16 **Supporting Information**

ABSTRACT: An interlaboratory comparison (ILC) was 17 organized with the aim to set up quality control indicators 18 19 suitable for multicomponent quantitative analysis by nuclear magnetic resonance (NMR) spectroscopy. A total of 36 NMR 20 data sets (corresponding to 1260 NMR spectra) were 21 produced by 30 participants using 34 NMR spectrometers. 22 The calibration line method was chosen for the quantification 23 of a five-component model mixture. Results show that 2.4 25 quantitative NMR is a robust quantification tool and that 26 out of 36 data sets resulted in statistically equivalent calibration 26 lines for all considered NMR signals. The performance of each 27 laboratory was assessed by means of a new performance index 28 (named $Q_{\rm p}$ -score) which is related to the difference between 29



the experimental and the consensus values of the slope of the calibration lines. Laboratories endowed with a Q_p -score falling within the suitable acceptability range are qualified to produce NMR spectra that can be considered statistically equivalent in terms of relative intensities of the signals. In addition, the specific response of nuclei to the experimental excitation/relaxation 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 analytical47 technique due to the possibility to derive a full uncertainty48 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. Measurement uncertainty is typically evaluated by three 79 so 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 quantification method. Such a model was followed in the first 87 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 data processing, and the evaluation procedure of each single 94 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 could be improved when a single operator processed all the 109 110 NMR spectra.

An interesting advantage of the NMR technique deals with h12 the possibility to suppress selectively one or more intense h13 signals with the consequent opportunity to enhance dramath14 ically the signal-to-noise ratio of weak signals. Typically, this h15 kind of experiment allows one to remove solvent signals thus h16 reducing the manipulation of the samples and avoiding the use h17 of large amounts of deuterated solvents. In routine experiments, h18 signal suppression can be simply obtained by implementing the h19 pulse sequence with a presaturation scheme consisting of a low power radio frequency pulse able to saturate a specific 120 resonance. 121

In principle, the introduction of the presaturation scheme 122 should not affect the quantitative NMR measurements. The 123 reproducibility of a single pulse experiment preceded by 124 presaturation of the solvent signal has been evaluated by 125 application of principal component analysis (PCA) to ¹H NMR 126 data in the framework of two interlaboratory comparisons.^{32,33} 127 PCA offers the advantage to estimate measurement reproducibility by easy visual inspection of the *scores* plot but quality 129 control indexes to be used as general reference parameters for 130 quality assessment of NMR spectra are still lacking. 131

With the aim to set up new quality control parameters 132 suitable for multi component quantitative NMR analysis as well 133 as for NMR fingerprinting methods, we have organized the first 134 Italian interlaboratory comparison according to the interna- 135 tionally agreed procedures ISO/IEC 17043:2010,³⁴ which 136 specifies general requirements for development and operation 137 of proficiency testing schemes, and ISO/IEC 17025:2005,³⁵ 138 which specifies the general requirements for the competence to 139 carry out tests and calibrations performed using standard 140 methods, nonstandard methods, and laboratory-developed 141 methods. The conventional statistical elaboration of data was 142 carried out according to ISO 13528:2005³⁶ and ISO 5725, parts 143 1-6.37 The analytical target of the comparison was the 144 quantification of analytes in a five-component model mixture 145 by the calibration curve approach and using the mL1M model 146 for uncertainty evaluation. Two different data elaborations were 147 considered: the first one was carried out by a single operator 148 who processed NMR spectra and developed calibration lines 149 with signal areas as input data, without referencing to any 150 standard molecule;³⁸ the second one was characterized by the 151 involvement of each participant in NMR spectra processing and 152 signal area calculation. In the second elaboration, signal areas 153 were scaled to a standard molecule and calibration lines were 154 developed by a specifically designed Web application. 155

In this paper, the comparison between results obtained by 156 the two data elaborations are discussed in terms of coefficient 157 of variation. The performance assessment in the second data 158 elaboration was carried out by means of the parameter (z- 159 score) usually considered as performance index in single 160 component quantifications as well as by means of a new 161 parameter, named Q_p -score, better suited for performance 162 assessment in multicomponent and fingerprinting analyses. 163 Moreover, a third index (NR), specific for each NMR signal, 164 was introduced to gain insights into the possible effects of the 165 acquisition parameters on signal intensities. 166

EXPERIMENTAL SECTION

Materials. 2-Methyl-2-(methylthio)propanal-O-(N- 168 methylcarbamoyl)oxime (Aldicarb, CAS No. 116-06-3, neat 169 purity 99.9%, Sigma-Aldrich, Milan, Italy), 2-methoxy-N-(2- 170 oxo-1,3-oxazolidin-3-yl)-acet-2',6'-xylidide (Oxadixyl, CAS No. 171 77732-09-3, neat purity 99.9%, Sigma-Aldrich, Milan, Italy), 172 O,S-dimethylphosphoramidothioate (Methamidophos, CAS 173 No. 102658-92-6, neat purity 98.5%, Sigma-Aldrich, Milan, 174 Italy), (2-dimethylamino-5,6-dimethylpyrimidin-4-yl)-N,N-di- 175 methylcarbamate (Pirimicarb, CAS No. 23103-98-2, neat purity 176 99.0%, Sigma-Aldrich, Milan, Italy), 3-(trimethylsilyl)-2,2,3,3- 177 tetradeutero-propionic acid sodium salt (TSP, CAS No. 24493- 178 21-8, 99% D, Armar Chemicals, Döttingen, Switzerland), 179 deuterium oxide (D_2O , CAS No. 7789-20-0, 99.86% D, 180 Sigma-Aldrich, Milan, Italy) were used for sample preparation. 181

167

182 Chemical structures of compounds are reported in Chart S1 in183 the Supporting Information.

Sample Preparation. Standard and test mixtures were 184 185 prepared under thermic and hygrometric control (20 \pm 5 °C, 186 40-60 R.H.%) by gravimetric method using a certified 187 analytical balance KERN ABT 100-5 M (KERN & Sohn GmbH, Balingen, Germany) with weighing range 1–101.000 188 189 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, corresponding to a confidence level of 95%, was considered 194 to determine extended uncertainties. 195

A solution made up of TSP in D_2O (20.33 \pm 0.29 mg/L) was 196 used to prepare six standard (labeled as A-E and Blank) and 197 one test (labeled as X) mixtures at the levels listed in Table S1 198 in the Supporting Information (in the range 0-500 mg/L). 199 Standard mixtures were used to obtain the calibration curves 200 considered to determine the concentration values of the test 201 mixture X. Mixtures were prepared by diluting stock solutions 202 to the desired concentration using class A glassware. NMR 2.03 tubes were filled in with 1.0 mL of the solution. 204

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

In order to choose the optimal recycle delay, T₁ values were 213 214 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 D_2O) at two different 218 magnetic fields, 9.4 T (400 MHz) and 16.5 T (700 MHz), 219 and two concentration levels, \sim 37 mg/L and \sim 600 mg/L. The 220 highest T_1 value (5.4 s, measured for M1 signal of a 37.4 mg/L solution of Methamidophos at 9.4 T) was taken into account to 221 222 set the recycle delay to 30 s. D₂O was not degassed before preparation of the solutions. Single component solutions and 223 test mixtures were prepared in the same laboratory using the 224 same batch of D₂O. NMR tubes were filled with 0.5 mL 225 226 solution, sealed, and delivered to the participants.

Data Acquisition and Processing. The NMR experiment 227 228 considered for the interlaboratory comparison consisted of a single 90° excitation pulse preceded by a selective presaturation 229 step. Even though it was organized before the publication of the 230 EUROLAB technical report on NMR method development 231 232 and validation,³⁹ this work produced results coherent with guidelines described therein. For each NMR tube, 5 spectra 233 were recorded to comply with conditions for repeatability 234 (measurements performed under the same operating con-235 ditions over a short period of time) considering the same NMR 236 237 tube, same spectrometer, same user, consecutive runs without removing the NMR tube from the magnet and to comply with 238 239 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) 3 245 consecutive runs per NMR tube (run 1, run 2, and run 3); (ii) 1 246 run per NMR tube delayed at least 24 h from the first session 247 (run 4); (iii) 1 run per NMR tube delayed at least 24 h from 248 the second session (run 5). It has been demonstrated³⁸ that 249 results obtained in repeatability conditions (considering only 250 data obtained by runs 1–3), in intermediate precision 251 conditions (considering only data obtained by runs 1, 4, and 252 5) and both conditions (considering data obtained by runs 1–253 5) can be safely considered as substantially equivalent. In the 254 present paper, calculation on all available replicates (runs 1–5) 255 will be described. More details on NMR data acquisition and 256 processing are reported in the Supporting Information.

Statistical Elaboration. Signal integrals were scaled to the 258 TSP integral and the corresponding (I_{signal}/I_{TSP}) values were 259 uploaded on a Web application specifically designed and 260 validated for data elaboration in agreement with internationally 261 accepted requirements.^{35–37} (I_{signal}/I_{TSP}) values were uploaded 262 reporting at least four decimal places. The five (I_{signal}/I_{TSP}) 263 replicates collected for each signal and for each NMR tube were 264 submitted to the Shapiro-Wilk test to ascertain their normal 265 distribution and to Huber, Dixon, and Grubbs tests for 266 identification of possible outliers. Throughout the paper, 267 Grubbs tests refer to application of both the classical Grubbs 268 test identifying one outlier and the double Grubbs test which 269 enables the identification of two outliers. Data identified as 270 outliers by all the four tests were not considered in successive 271 steps. Data derived from standard mixtures A-E and Blank 272 were used to plot (I_{signal}/I_{TSP}) versus analyte concentrations 273 and to develop an equation for the calibration line by least- 274 squares linear regression. The equation of general formula y = 275ax + b (with $y = (I_{signal}/I_{TSP})$ and x = concentration as mg/L) 276 was used to calculate concentration values of analytes in test 277 mixture X. Then, the 5 concentration values calculated for the 278 test mixture X were submitted to the Shapiro-Wilk test to 279 ascertain their normal distribution and to Huber, Dixon, and 280 Grubbs tests for identification of possible outliers. After 281 removing outliers, calculated concentrations were used to 282 determine the mean concentration values and the correspond- 283 ing standard deviations which were considered as intra- 284 laboratory uncertainties of the method. Results from all 285 participants (36 sets of results from 34 NMR spectrometers) 286 were submitted to data elaboration for proficiency test and for 287 determination of the assigned values for analytes in mixture X. 288 The lack of official qNMR analyses for this case study 289 prompted us to determine assigned values as consensus values 290 from participants.³⁴ Thus, for each analyte, according to the 291 flowchart suggested by Horwitz,⁴⁰ the 36 standard deviation 292 values were submitted to the Cochran test (provided that all of 293 the 5 replicates of mixture X successfully passed the above- 294 mentioned tests for outliers) with the aim to identify and 295 remove outliers for successive calculations. In turn, mean 296 concentration values from data sets which passed successfully 297 the Cochran test were submitted to Grubbs tests with the aim 298 to further refine the quality of the results. The remaining sets of 299 data were submitted to the Shapiro-Wilk test to ascertain the 300 normal distribution of the population (data were always normal 301 distributed after refinement by the Cochran and Grubbs tests) 302 and were used to calculate, for each analyte in test mixture X, 303 the assigned concentration value, the interlaboratory standard 304 deviation, the coefficient of variation (CV%), and the 305 reproducibility limits. 306

307 RESULTS AND DISCUSSION

³⁰⁸ **Performance Assessment for Single Component** ³⁰⁹ **Quantitative NMR Measurements.** Among the quantifica-³¹⁰ tion approaches available for NMR spectroscopy,⁴ the ³¹¹ calibration line method was chosen in this work as it allows ³¹² for identification of a theoretical line to be taken as reference in ³¹³ performance assessment. Moreover, this method has a general ³¹⁴ applicability in analytical chemistry and has the advantage to ³¹⁵ nullify the effects of nuclei relaxation on quantitative accuracy, ³¹⁶ provided that all the acquisition parameters are kept constant ³¹⁷ for standard and test solutions.⁴ Thus, it is expected that ³¹⁸ systematic errors deriving from hardware features or from the ³¹⁹ set of acquisition parameters should be minimized.

A first statistical data elaboration of the ILC was carried out 320 321 by a single operator who processed NMR spectra (Fourier 322 transformation, phase and baseline correction, signal integra-323 tion) and obtained calibration lines with no scaled signal areas 324 as input data.³⁸ In a second data elaboration, NMR data 325 processing was carried out by each participant and signal areas 326 were scaled to the TSP area. Therefore, the main difference 327 between the two elaboration approaches relays on different processing conditions. Results of both elaborations are 328 329 summarized in Table S3 in the Supporting Information 330 where assigned concentration values along with the corre-331 sponding standard deviations, coefficients of variation, and 332 reproducibility limits are reported. It is apparent that changing 333 the processing conditions of the NMR spectra, from "one 334 operator-all NMR data sets" to "one operator-one NMR data 335 set", has a little impact on the final result in terms of mean 336 value. Conversely, standard deviations (and consequently the 337 related coefficients of variation and reproducibility limits) are 338 affected by the different NMR processing conditions. 339 Notwithstanding the deterioration of their quality in terms of 340 coefficient of variation (CV%), these results are quite satisfactory if this test is considered as a confirmatory method 341 342 for organic residues and contaminants. Indeed, according to the 343 European Commission decision concerning the performance of 344 analytical methods and the interpretation of results,⁴¹ the 345 interlaboratory coefficient of variation (CV%) for repeated 346 analysis of a reference material, under reproducibility 347 conditions, shall not exceed 5.7% for concentration values 348 higher than 1000 ppm, according to the Horwitz equation:

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

349 where *C* is the mass fraction expressed as a power of 10 (e.g., 1 350 mg/g = 10^{-3}). Being the concentration values considered in 351 this work are lower than 500 mg/L, the highest obtained CV% 352 value of 4.9% indicates that single excitation pulse preceded by 353 selective presaturation of the solvent is a reliable NMR 354 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 all quantitative results is the *z*-score, which is defined as

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

³⁶² where C_i is the mean concentration value determined by the *i*th ³⁶³ data set, \overline{C} is the assigned concentration value, and σ is the

interlaboratory standard error, all referred to as a single NMR 364 signal. Satisfactory performance is indicated by $|z| \leq 2.0$, 365 questionable performance is obtained when 2.0 < |z| < 3.0, 366 while $|z| \geq 3.0$ indicates unsatisfactory performance. In the 367 latter case, suitable actions are required to identify and to solve 368 the analytical problems. 369

Figure 1 shows the z-scores of Aldicarb quantification by the $_{370}$ f1 NMR A1 singlet. It is apparent that, even though results of 10 $_{371}$



Figure 1. *z*-score for quantification of Aldicarb by means of A1 signal (green, $|z| \le 2.0$; yellow, 2.0 < |z| < 3.0; red, $|z| \ge 3.0$. 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 372 value, the quality of the result was satisfactory for 35 sets of 373 NMR data and only 1 unsatisfactory performance was 374 registered. Very similar results were obtained using each of all 375 other NMR signals (Supporting Information, Figures S2–S8). 376 High-performance quantifications are obtained also when 377 signals different from singlets were taken into account (as in 378 the case of M1 and O2 where a doublet and a group of signals 379 were considered, respectively). It is worth noting that 380 performance in terms of result quality was not affected by the 381 magnetic field, hardware configuration, manufacturer, and 382 production year of the spectrometer. These findings highlight 383 the robustness of NMR spectroscopy when calibration curve 384 approaches are adopted. 385

The z-score represents a satisfactory indicator for perform- 386 ance assessment in single component analyses, but it cannot 387 account for performance assessment in multicomponent 388 analyses because a single z-score refers to only a single 389 quantification measurement. Thus, for performance assessment 390 in fingerprinting measurements and quantitative multicompo- 391 nent analyses, the introduction of indicators more appropriate 392 than the z-score is desirable. 393

Basics of Quantitative NMR. Before discussing the new 394 quality control parameters proposed by us in performance 395 assessment for fingerprinting measurements and quantitative 396 multicomponent analyses, recall of the basic equation of 397 quantitative NMR is advisible (eq 1). 398

$$I = kn \tag{1}_{399}$$

Equation 1 provides the direct proportionality between the 400 number of moles (n) of nuclei generating a signal and the 401 intensity (I) of the same signal with a proportionality constant 402 k being the spectrometer constant which remains the same for 403 all resonances in a NMR spectrum.⁴ 404



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 \bullet ; green, $|Q_p| \le 2.0$; yellow, 2.0 < $|Q_p| < 3.0$; red, $|Q_p| \ge 3.0$.

Let us consider, in a NMR spectrum, the signal (a) having 406 intensity I_a generated by specific protons belonging to the 407 analyte of interest and the signal (r) having intensity I_r 408 generated by specific protons in a reference compound. 409 Applying eq 1 to I_a and I_r gives

$$I_a = kn_a$$

$$I_r = kn_r$$

4

42

410 Hence the ratio $(I_a/I_r) = (n_a/n_r)$ (eq 2) is independent from the 411 proportionality constant *k* and, as a consequence, it does not 412 depend on the spectrometer. Thus, taking the methyl protons 413 signal of TSP as reference signal, all of the calibration lines 414 obtained plotting (I_a/I_{TSP}) versus analyte concentration (*C*) 415 should be independent from the spectrometer and statistically 416 equivalent to each other. In other words, all the participants to 417 an ILC should develop equivalent calibration lines

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

419 where intercept *b* should have a null value due to the fact that 420 no signal is generated if no nuclei (C = 0 mg/L) are contained 421 in the mixture. Thus, eq 3 can be rewritten as

$$\left(\frac{I_{a}}{I_{TSP}}\right) = \left(\frac{n_{a}}{n_{TSP}}\right) = \left(\frac{\frac{m_{analyte}}{M_{analyte}}}{\frac{m_{TSP}}{M_{TSP}}}N_{a}\right) = aC = a\frac{m_{analyte}}{V}$$

$$(4)$$

423 where m_{analyte} is the mass of the analyte, m_{TSP} is the mass of 424 TSP, M_{analyte} is the molar mass of the analyte, M_{TSP} is the molar 425 mass of TSP, N_{a} is the number of protons generating the signal 426 (a), N_{TSP} is the number of methyl protons (nine) generating 427 the reference signal, and V the solution volume. 428 Equation 4 can be rearranged into

$$\left(\frac{\frac{m_{\text{analyte}}}{M_{\text{nalyte}}}N_{\text{a}}}{\frac{m_{\text{TSP}}}{M_{\text{TSP}}}N_{\text{TSP}}}\right) = a\frac{m_{\text{analyte}}}{V}$$
(5) 42

From eq 5 the theoretical value that slope must assume for a 430 given TSP concentration can be extracted: 431

$$a_{\text{theoretical}} = \frac{M_{\text{TSP}}}{M_{\text{analyte}}} \frac{N_{\text{a}}}{N_{\text{TSP}}} \frac{V}{m_{\text{TSP}}} = \frac{M_{\text{TSP}}}{M_{\text{analyte}}} \frac{N_{\text{a}}}{9} \frac{1}{C_{\text{TSP}}}$$
(6) 432

The need to harmonize NMR protocols prompted us to 433 propose a new parameter suited for checking the equivalence of 434 the calibration lines. Such a parameter will be shown to 435 represent a quality control index of the NMR spectra to use in 436 fingerprinting applications and multicomponent NMR quanti- 437 fications.

Quality Control Parameters for Performance Assess- 439 ment in Fingerprinting Measurements and Quantitative 440 Multicomponent Analyses. In order to assess the laboratory 441 performance in multicomponent analyses without considering 442 as many *z*-scores as the number of analytes, we propose a new 443 parameter, named Q_p -score, accounting for participant perform- 444 ance as the result of instrumental adequacy and operator skill. 445 Knowing that, for each signal, calibration lines developed by 446 each participant must be equivalent to each other, let us define 447 the indicator of the line equivalence Q_p as 448

$$Q_{\rm p} = \frac{a_i - \bar{a}}{\sigma_{\rm slope}} \tag{7}_{449}$$

where a_i is the slope of the calibration line determined by the 450 *i*th participant, \overline{a} is the consensus slope value, and σ_{slope} is the 451 interlaboratory standard deviation on slopes, all referred to a 452 single NMR signal. The values \overline{a} and σ_{slope} are determined using 453 a_i successfully passing the Huber test. By an analogous 454 reasoning followed for the z-score, performance assessment 455 by the Q_p -score is considered satisfactory when $|Q_p| \leq 2.0$, 456

Р

	Signal									
	A1	A2	A3	M1	01	O2	P1	P2		
articipant label										
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, $|Q_p| \le 2.0$; yellow, 2.0 < $|Q_p| < 3.0$; red, $|Q_p| \ge 3.0$.

⁴⁵⁷ questionable when 2.0 < $|Q_p|$ < 3.0 and unsatisfactory when $|Q_p|$ ⁴⁵⁸ \geq 3.0.

In the case study of Aldicarb quantification by NMR signal 459 460 A1, Huber tests applied to the 36 slope values gave 11 outliers, the 5 lowest and the 6 highest values. The 25 remaining values 461 resulted in a normal distribution after the Shapiro-Wilk test and 462 were considered for the calculation of \overline{a} and σ_{slope} . The values of 463 and $\sigma_{\rm slope}$ were 0.0340 L/mg and 0.0032 L/mg, respectively. 464 ā 465 Concerning the experimental intercept values, the population 466 was not normal and was too scattered so that iterated Huber test gave meaningless results (all values were identified as 467 outliers). The mean value of the intercept was -0.048, and the 468 related standard deviation was 0.513 indicating that the null 469 value can be well considered as the experimental intercept. 470

These results give y = 0.0340x as the consensus equation for 471 the calibration line but do not yet demonstrate the statistical 472 equivalence of the calibration lines. In order to evaluate the 473 statistical parallelism, and then the equivalence of the 474 calibration lines, all possible slope pairs were submitted to 475 the paired t test with 95% confidence level. Computational part 476 of the test consists of calculation of parameter $t_{\rm slopes}$ as the 477 difference between two slopes divided by the standard error of 478 the difference between the same two slopes.⁴² Then, t_{slopes} was 479 compared with Student's t at the desired confidence level 480 (95%) to evaluate whether the null hypothesis was supported, 481 482 that is whether no relationship between two data sets existed. If 483 the slope obtained by one data population is significantly 484 different from that generated with another (and independent) 485 data set, then $t_{slopes} > t$ or else $t_{slopes} \le t$, and the two slopes can 486 be considered statistically equivalent. Results of the paired t 487 tests applied to all possible slope pairs are summarized in 488 Figure 2 where statistically equivalent lines are cross-linked by 489 black circles. For instance, participant P11 produced a 490 calibration line which is equivalent to those produced by

participants P27, P07, P20, P19, P08, P13, P35, P26, P02, P33, 491 P23, P05, and P18. 492

It is apparent from Figure 2 that the slopes of 26 calibration 493 lines (bordered by black dashed lines) are statistically 494 equivalent. Of these 26 lines, 23 were characterized by $|Q_p|$ 495 lower than 2, i.e., in the range of satisfactory performance 496 assessment. Moreover, with the exception of participant P10, 497 the highest number of simultaneous coincidences (7–14) was 498 recorded for participants endowed with very low Q_p -scores 499 (ranging from -0.63 to 0.43, bordered by red dashed lines in 500 Figure 2).

The statistical equivalence of the calibration lines is in 502 agreement with the theoretical treatment described above. It 503 represents the experimental evidence that, for a given TSP 504 concentration, slopes assume a certain value depending on the 505 signal and not on the spectrometer constants. Once defined, 506 the concentration range of the analytes, slopes associated with 507 satisfactory Q_p -scores indicate that the corresponding NMR 508 spectra were recorded and processed under similar conditions. 509 Deviation from the consensus value of the slope is explained in 510 terms of hardware reliability, acquisition and processing 511 parameters. Therefore, Q_p-score represents a quality control 512 index which accounts for hardware functioning conditions and 513 operator skills. It is important to point out that questionable 514 and unsatisfactory Q_p-scores did not prevent successful single 515 component quantifications as the latter depend only on the 516 quality of the calibration line. Indeed, good fitting in the linear 517 regression allows for a good performance in terms of z-score 518 but it does not account for deviation of the slope from its 519 theoretical value. 520

Given that the Q_p -score is a quality parameter of the NMR ⁵²¹ spectrum as a whole, it can be expected that, as far as ⁵²² multicomponent analysis is concerned, Q_p -score based perform- ⁵²³ ance assessment of a laboratory should be almost independent ⁵²⁴ from the considered signal. This is indeed the case, as clearly ⁵²⁵ ß

signal	A1	A2	A3	M1	01	O2	P1	P2
$M_{\rm analyte} \ (g/{ m mol})$	190	190	190	141	278	278	238	238
N_{a}	6	3	1	3	3	3	3	3
$a_{\rm theoretical} \times 10^2 \; ({\rm L/mg})$	2.97	1.48	0.49	2.00	1.01	1.01	1.19	1.19
$\overline{a} \times 10^2 \; (\text{L/mg})$	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
${}^{a}M_{\text{TSP}} = 172.27 \text{ g/mol; } N_{\text{TSP}}$	$= 9; C_{TSP} = 20$.33 mg/L.						

Table 1. NR Values	(%) Calculat	ed for All NMR	Signals As L	abeled in T	Table S2 in the	e Supporting	Information
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t1

s26 demonstrated by inspection of Figure 3, where Q_p -scores s27 obtained by each participant by considering each of the NMR s28 signals selected for this study are reported. Apart from the s29 variations of the Q_p -scores falling in the proximity of the s30 limiting value ± 2 , the performance category ($|Q_p| \le 2.0, 2.0 < |$ s31 $Q_p| < 3.0$ and $|Q_p| \ge 3.0$) is retained for all considered signals. s25 These findings are in good agreement with the high s33 reproducibility of ¹H NMR experiments evaluated by PCA in s34 previous studies.^{32,33}

The introduction of Q_p -score paves the way to validation of 535 536 multicomponent quantification methods, of great importance 537 for fingerprinting and profiling applications. In fact, such validation procedures might be carried out in the future by an 538 539 interlaboratory comparison where laboratory performance 540 could be preliminarily assessed developing calibration lines 541 for any arbitrary compound mixture. Once a Q_p acceptability 542 range is fixed (for instance, $|Q_p| < 1$), all laboratories within 543 such a range will be qualified to produce NMR spectra of a given mixture that are statistically equivalent in terms of relative 544 545 intensities of the signals. In other words, for a given set of 546 acquisition parameters, laboratories gaining satisfactory $Q_{\rm p}$ scores will be accredited to record NMR spectra on every kind 547 548 of mixture, thus allowing for pooling of NMR data in suitable databanks. 549

It is worth noting that, in real experiments, a deviation from 550 551 theoretical slope is expected due to the specific response of the 552 nuclei to the experienced excitation/relaxation conditions 553 during spectrum acquisition. Such a response depends on several factors including (i) hard excitation pulse which must be 554 uniform throughout all the spectral width; (ii) proximity of the 555 556 signals to the offsets; (iii) recycle delay, which must be long 557 enough to allow for complete magnetization recovery of all 558 nuclei; (iv) energy exchange effects (NOE, spin diffusion, etc.) 559 introduced by soft pulses. Therefore, in any interlaboratory 560 comparison the consensus slope may differ from the theoretical one as an effect of the specific set of acquisition parameters. 561

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

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

567 NR calculated for all signals considered in this study are 568 reported in Table 1.

NR values for the various signals ranged from -18.2% to 570 6.2% indicating that signals are not affected to the same extent 571 by the used acquisition parameters. Moreover, NR values were 572 different also for signals generated by inequivalent nuclei in the 573 same molecule.

In the present case, NR represents an index of the response 575 of the nuclei submitted to a NMR experiment characterized by 576 a single 90° excitation pulse preceded by a selective

presaturation step with the specific set of acquisition 577 parameters. In our opinion, among the above-mentioned 578 factors affecting the nuclei response, energy exchange effects 579 introduced by soft pulses can be considered the most relevant 580 to interpret the NR values obtained in the present study. 581 Energy exchange effects are certainly operative in the 582 acquisition condition characterized by a selective pulse acting 583 during the long recycle delay (30 s). The other factors are 584 thought to affect NR values only marginally because possible 585 incorrect setting of the pulses (factors i and ii) will give random 586 contributions averaged to almost null deviation of the 587 calibration line and because the adopted recycle delay (longer 588 than 5 times the highest measured T_1) ensures complete 589 recovery of the magnetization (factor iii). Anyway, a deeper 590 study on factors affecting the nuclei response to experimental 591 acquisition conditions to give the NR values reported in Table 592 1 requires further NMR experiments. This is out of the scope of 593 the present paper. 594

This study introduces a new quality control parameter, $Q_{\rm p}$ - 596 score, suitable for harmonization of fingerprinting protocols 597 and quantitative multicomponent analysis. Such a parameter, 598 that was designed considering consolidated internationally 599 agreed statistics, represents an unbiased evaluation tools for 600 NMR method validations.

The Q_p -score accounts for laboratory performance in terms 602 of both instrumental adequacy and operator skill and enables 603 laboratories to pooling of NMR data in suitable databanks. 604 Moreover, Q_p can be valuable for the development of 605 multilaboratory metabolomic platforms. In fact, it was shown 606 that participants having a Q_p-score in a suitable acceptability 607 range are able to produce NMR spectra of a given mixture that 608 can be considered statistically equivalent in terms of relative 609 intensities of the signals. Another practical use of Qp-score 610 consists of the entitlement of laboratories endowed with 611 acceptable $|Q_p|$ values to carry out quantifications by using 612 relative intensity of the signal of interest after fitting with the 613 consensus calibration line deriving from the interlaboratory 614 comparison. For instance, in suitable networking conditions, 615 equivalent calibration lines could be shared to enable different 616 laboratories to carry out quantitative analyses without wasting 617 time in calibration steps, with a consequent increase of 618 productivity. 619

Since basic equations of quantitative NMR are independent 620 from the type of solvent, it can be expected that performance 621 assessment by Q_p -score applies also to experiments carried out 622 in solvents others than D_2O (for instance, in organic solvents 623 such as CDCl₃ widely used in lipidomics and in complex 624 mixtures such as biofluids which are mostly used in their native 625 state). 626

Another parameter, NR, has been proposed, which is related 627 to differences between the theoretical and the consensus slopes 628

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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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652 Author Contributions

653 The manuscript was written through contributions of all 654 authors. All authors have given approval to the final version of 655 the manuscript.

656 Notes

657 The authors declare no competing financial interest.

⁶⁵⁸ ⁴⁻³³The complete list of the affiliations is reported in the ⁶⁵⁹ Supporting Information.

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