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Review

Quantitative NMR in synthetic and combinatorial chemistry

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Abstract

The applications of quantitative NMR to synthetic organic chemistry are reviewed with taking into account both the small libraries (100–150 compounds) and the single, well-characterized substance. The precision and accuracy which are obtained with state of the art instrumentation – both around 1% – rival with other classical tools of quantitative analytics, and qNMR does not require a specific method setup or a standard of the same substance. This characteristic makes it the method of choice in an environment where many different molecules are investigated and reliable quantification is required. NMR may effectively replace other standard characterization tools, such as CHNS analysis, or even complex, multi-determination results as commonly required for the assessment of absolute purity or strength of a substance, when no specific standard is available. Finally, because of the high precision and intrinsic accuracy, quantitative NMR appears the ideal reference method for the validation of other, more rapid, generic techniques for quantitative analysis.

Keywords: Chemical libraries; Standards; Absolute purity; Quantitative NMR; Elemental analysis

Contents

1. Introduction

The determination of concentration and purity of a molecular species are two strictly related tasks in Chemistry. If purity is defined as the amount of one single molecular species contained in the total available amount of substance, then it can be obtained from the concentration of the same molecular species and other simply measurable quantities such as mass or volume. In classical analytics, determination of concentration requires a specific method and a reference standard, both of which are generally available only for very well investigated compounds. However, in frontline organic chemistry (combinatorial, parallel, high throughput synthesis) a more

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practical definition of purity is often used: e.g. the fraction of HPLC-UV signal assigned to a molecular species with respect to the total signal obtained with this technique. The error contained in this definition is considered acceptable if the obtained purity exceeds a given threshold (typically 90–95%). Early eluting (solvent front) or column-retained compounds are not considered in this approach, and species with a weak (water, salts, solvents, etc.) or strong extinction coefficient are under- or overestimated, respectively [1]. Good practice requires an independent assessment in order to exclude excessive contamination by solvents, salts or other contaminants. This is typically obtained with CHNS elemental analysis. Recently, two HPLC detectors (Evaporative Light Scattering, ELSD, and Chemiluminescent Nitrogen specific, CLND) have shown the capability to determine the molecular concentration in a generic way, i.e. without the need of a specific standard for each analyte, with fairly acceptable results [2,3]. These techniques have been critically discussed very recently [1,4,5] and will not be reviewed here.

In this review we focus on quantitative NMR as an alternative method for the determination of absolute purity (and thus absolute concentration), with the intent to convince the reader that this is a simple, affordable tool which can reach the goal with very limited experimental effort and with reduced sample consumption, leading to high quality results. Once established in the laboratory toolbox, this technique may contribute to expand quality and efficiency with direct impact on: measuring true reaction yields, determining purity of well-characterized molecules, rapidly profiling small chemical libraries, assessing other analytical techniques with highly reliable comparative data.

We will mainly describe applications of proton NMR because this nucleus is the most generally used due to its sensitivity and widespread presence in organic molecules even though practically all the NMR active nuclei can be employed.

2. Principles of NMR quantification

Under appropriate conditions, the area of each NMR peak is directly proportional to the number of the corresponding nuclei. Thus, at variance with other techniques, the response factor is not dependent on the molecular structure, and relative concentrations may be assessed with calibrating on a single, well-characterized standard having a molecular structure different from that of the analyte. Therefore, this technique is ideally suitable for generic quantitative detection of organic molecules, where the presence of ^{1}H nuclei is ubiquitous.

The availability of high field instruments in conjunction with improvements in probe design and electronic performance have considerably increased sensitivity/resolution and eliminated artifacts which reduced precision and limited applicability of quantitative NMR determinations until about 15 years ago. Introduction of digital acquisition ("digital signal processing" according to Varian terminology) [6–8] combined with optimization of pre-acquisition delays [7,9,10] has reduced amplitude errors in the first few points of the FID thus improving baseline performance. Linear prediction methods can also be applied to reconstruct the beginning of the FIDs [11]. In addition, digital filters overcome problems bound to conventional analog filters [12] and afford exactly linear phase response and constant amplitude response over the frequency band of interest [7]. In any case, the intensity and phase response should be checked using a sample with a single intense line and varying its position in the spectral window. If the response is found *non-linear* but reproducible in the range of interest, a correction function can be introduced [13]. This takes into account the off-resonance effect due to *non-constant* excitation of the rf pulse, as well.

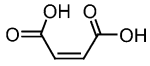
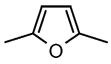
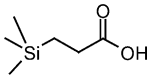
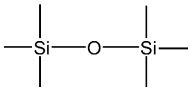
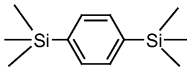
The optimization of experimental parameters is a well-known topic (e.g. [14–16]) and the source and magnitude of errors has been thoroughly discussed by Griffiths and Irving [17]. The use of a long pulse interval (e.g. 30–60 s) to warrant full signal relaxation, and the need for very high signal to noise levels set practical limitations on the sensitivity of the method; nevertheless, for modern high field instruments with state of the art electronics and probes, precision is about 1% in a working range around 5–20 mM. This level of solubility for both standard and test sample in the solvent of choice is a requirement, as well as the presence of well-resolved signals in the spectrum.

Probably the main limitation of qNMR is the need of human intervention during processing operations that closely influence integral values (i.e. phasing, choice of the integrated signals and integral tails setting). Software commands are available which reduce at minimum subjective decisions on part of the operator; however, according to our experience these tools perform less well than manual processing by a skilled operator. In fact, especially if the timing of data acquisition has been optimized so that first-order phase shift is zero [7,9,10] and only zeroth order correction is applied, spectra phasing can be performed easily and reproducibly.

NMR lines are Lorentzian shaped and therefore the integration tails should extend at least 20–30 times the line-width each side of the peak to include 99% of the area. Of course, this is not always applicable due to neighboring signals. The effect on accuracy is minimized when the integral ratios between the internal standard and the analyzed molecule signals is calculated using identical criteria to select the tails. For accurate results ^{13}C satellite peaks should be included in the integration tails but they can be excluded in both integral regions for the above considerations. Choice of the integrated signals is a critical step that can strongly affect the result especially in the case of crowded spectra with impurity signals overlapping resonance of the test molecule. Whenever possible, isolated and sharp peaks must be chosen and broad signals must be discarded unless an adequate integration tail can be added without including nearby peaks.

The standard deviation deriving from the average of the integrals of the chosen signals inside every single spectrum is an intrinsic measure of integration accuracy and also provides

Table 1
Proposed standards for quantitative NMR

Name	Structure	Comments	Reference
Maleic acid		Not chemically inert. Easily weighable solid. Soluble in water	[26,18]
2,5-Dimethylfuran (DMFu)		Volatile, easily removable	[29]
Trimethylsilylpropionic acid		Easily weighable solid. Soluble in water. Unstable in solution	[26]
Tetramethylsilane (TMS)		Volatile, easily removable. Soluble in organic solvents	[36]
Hexamethyldisiloxane (HMDS)		Volatile, easily removable. Soluble in organic solvents	[33,34]
Bis-1,4-trimethyl-silyl-benzene		Easily weighable solid. Soluble in organic solvents	[13,25]
Dimethyl-sulfone		Soluble in water and in organic solvents	[22]

information about the presence of impurities. When signals of impurities are perfectly superimposed to resonances of the target molecule the chosen integrals are not generally self-consistent. In this case, only the lowest values must be considered for purity calculation.

Preparation of the NMR sample by accurately weighing both test compound and standard ensures high precision and accuracy and this is the method currently recommended by USP [24]. In a routine environment, a more convenient procedure, which only requires the weight of the test compound and a volumetric addition of a stock solution of the standard with a micropipet, leads to acceptable results [25]. For the characterization of libraries, this procedure is easily automated with the use of a liquid handler and of stock solutions for the test compounds [13]. Stability of the standard solution is a requirement, or the need for frequent re-preparation of this solution would eliminate the advantage of volumetric rather than gravimetric addition of the standard.

3. Selection of an appropriate standard

The choice of an appropriate internal standard is instrumental for the development of a method with wide applicability and good precision. Ideally, the reference compound must be highly pure, soluble in the solvent of choice, stable for long time under these conditions, and it should not react with any of the test compounds; moreover, it should have an intense singlet in a usually signal free region of the NMR spectrum. Silanes have been used as reference standards in NMR; however, most simple silanes (i.e. TMS, hexamethyldisilane) are highly volatile liquids, and their use for quantitative determinations requires a secondary standard. Several compounds have been proposed and each has its own characteristic. These are summarized in Table 1 and a discussion on the corresponding *pros and cons* has been reported [13]. Probably the choice should take into account the actual needs of the experimenter: if the NMR sample must be recovered, then a volatile standard is the preferred choice; if accurate measurements are needed, then a weighable solid is the best choice. In every case, the signal(s) of the standard should not interfere with those of the sample.

A very interesting alternative to the internal standard is the ERETIC method, which uses a reference electronic signal for the quantification of proton resonances. Precision of about 1% has been obtained and the calibrated signal is claimed stable for more than 1 month, without a need for recalibration [19]. Unfortunately, this highly versatile method has not yet received the attention it deserves, probably because commercially available spectrometers do not have the required hardware by default or need some re-cabling to perform the ERETIC sequence. Potential applications are, indeed, very broad and recently its usage has been extended to deuterium NMR [20] and 2D-spectroscopy [21]. The advantage in sample preparation, the lack of sample contamination and its applicability to imaging work make the ERETIC method by far the most suitable for generic quantification purposes. Hopefully, its use will gain acceptance in the analytical community during the next years.

4. Accuracy and precision of qNMR determination

In a very thorough investigation, Maniara et al. [18] have clearly established that precision and accuracy well within 1% are achievable with quantitative NMR if the sample

concentration is maintained above 20 mM. With an apparatus provided with modern electronics, Pinciroli et al. [25] could demonstrate a similar performance at a concentration of 10 mM or slightly lower. In particular, data reproducibility on a large set of compounds (248 different molecules) was witnessed by a distribution of measurement differences with a standard deviation of 1.3%. This figure improved to 0.9% with a restricted set of molecules (103) where a carefully optimized procedure for sample preparation was applied. A comparative study on NMR peak integral consistence between laboratories has been published [35], and the results agree with the above conclusions. Average intra-laboratory precision was around 1%, and inter-laboratory values varied between 1.2% and 2%. Finally, in an accurate study on agrochemicals, Wells et al. [22] state that "qNMR analysis of agrochemicals in this paper is both more accurate and more precise than standard HPLC methods".

Even with ^{31}P spectroscopy, precision of 1% or better can be achieved with the use of coaxial inserts containing a reference standard [30], in spite of the new source of error given by the variation of relative insert to sample volume. Interestingly, a similar performance is also reported for the ERETIC method which uses a reference electronic signal for absolute calibration [19].

Determination of accuracy is largely biased by the knowledge of the purity of the test substance as assessed with an independent method [18]. If this is minimized with the choice of a highly pure reference standard, then accuracy is equivalent to method precision. In practice, it is advisable to verify accuracy with well-known compounds that may be purchased in highly purified form (e.g. acetanilide, caffeine).

If low concentration is mandatory, as with chemical libraries where compound availability is scarce, precision of quantification deteriorates, because of decreasing signal to noise ratio, needing impractically long measurement time for compensation. In our experience with 400–500 MHz instruments and non-refrigerated ^{1}H-probes, qNMR may be applied to solutions with concentration as low as 1 mM if a precision of 5% is acceptable. Better results would require higher field instruments or more sensitive cryoprobes.

5. Purity and concentration of small libraries

Though NMR is heavily applied to combinatorial chemistry [31,32], very few studies on the application of qNMR have appeared in this area. To our knowledge, qNMR was applied to the characterization of libraries in just a few cases [1,13,39] whereas in other studies was used for special purposes [33,34] or cited as a potential method for purity determination without a systematic application to combinatorial samples [1,36,37].

Automatic NMR spectra collection of a large set of samples is nowadays possible with the use of sample changers or liquid handlers in conjunction with flow probes [27,33,38] and the main reason for the rare application of qNMR is the need for spectra interpretation. In particular, signals of the target molecules must be differentiated from those of impurities, in order to obtain reliable peak integral values. This is considered a highly demanding step for chemical libraries, because of the high level of contaminants often encountered in these compounds [1]. However, if NMR is used as a quality control method during the optimization of the synthetic procedure [39], spectral features of common scaffolds and building blocks are usually well assigned, and such a preliminary experience highly expedites the task if the same person is involved. In our experience, a well-trained spectroscopist can fully characterize (both qualitatively and quantitatively) a small chemical library (100–150 compounds) in less than one working week [13]. Nevertheless, this is the rate-limiting step of the method (in conjunction with the need for manual data processing) and a tool for automatic signal assignment would speed up the whole process enormously. There is a lot of effort in this area [28] but no fully reliable software exists for this purpose. When a large number of molecules is investigated, and all derive from the same scaffold, it is advisable to use one or more peaks from this scaffold region for the quantification purpose. These signals are usually maintained in a narrow range of chemical shift and are thus easily assigned, with a high impact on the efficiency of the entire process. A very thorough discussion about the various methods of data presentation that facilitate identification of common features in chemical libraries is reported in Ref. [27]. As these authors clearly show for a small library of imides (88 compounds), data presentation is essential in order to expedite data analysis and interpretation. Using the tools discussed in Ref. [27], the spectroscopist does not need full spectrum assignment for all molecules and may proceed with the quantification step very rapidly, as long as one or two distinctive peaks have been identified in all (or most of) the spectra.

In a study carried out on 308 samples belonging to three small chemical libraries, each one representing a different structural class [13], the purity distribution as obtained with HPLC-UV–MS was compared with absolute concentration data from NMR. The results are shown in Fig. 1, where NMR purity is reported as % of the nominal concentration (concentration that was estimated by the library preparer for the solutions) otherwise called strength. The distribution of NMR strengths, which varies from 5% to 213%, is much broader than the corresponding LC-UV distribution because this latter is just a relative purity measured upon assuming the sum of all LC-UV peaks equal to 100%. An equivalent error would be introduced in all data generated by analyzing these solutions if concentrations were not corrected. LC-UV purity distribution indicates good quality of the investigated molecules, but cannot be used as a concentration correction factor.

Pharmacological data of a few active compounds belonging to this set confirmed the value of this approach. Once these molecules were synthesized in a pure form to confirm biological activity, the IC_{50} measured on these new

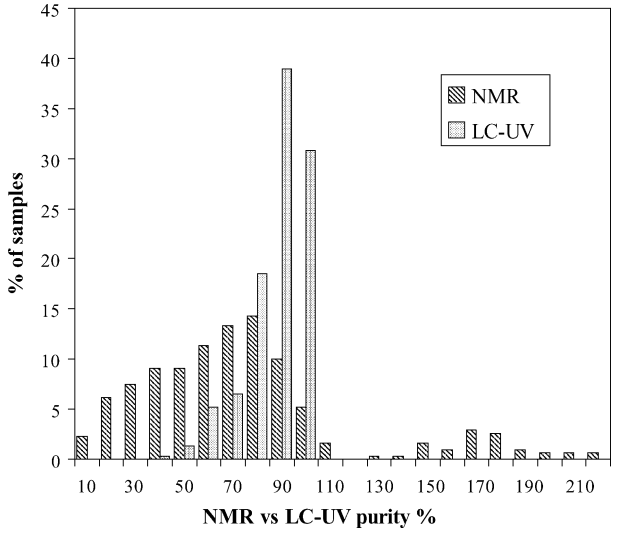


Fig. 1. Comparison of NMR purity distribution (% of the nominal concentration) vs. the HPLC-UV purity distribution of 308 molecules belonging to three different chemical libraries. Both distributions are reported in 10% steps. Reprinted from Ref. [13] with permission from American Chemical Society.

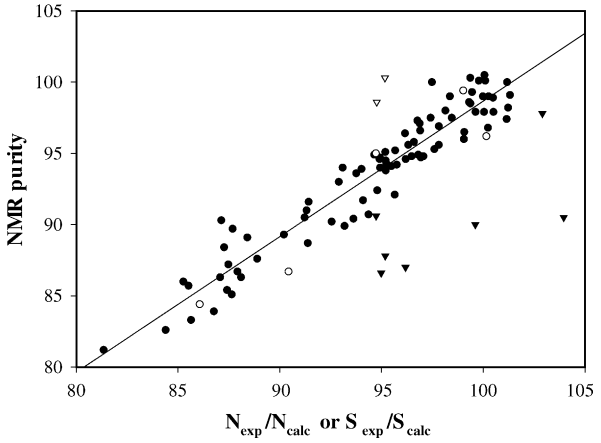


Fig. 2. Correlation between ^{1}H NMR purity and estimates from elemental analysis. Filled circles denote data for N_{exp}/N_{calc}, empty circles are data on S_{exp}/S_{calc}. Triangles are used for data which were not included in the correlation analysis: either because of poor ^{1}H NMR estimate (empty triangles, impurities signals overlapping integrated signals), or because of apparent presence of nitrogen-containing contaminants (filled triangles). Reprinted from Ref. [25] with permission from American Chemical Society.

samples were in a good agreement with the values obtained with combinatorial chemistry solutions after correction with NMR strength.

6. Alternative to elemental analysis for well-characterized molecules

Elemental analysis results are often used as a crude estimation of purity, since the ratio of measured vs. calculated amount of hetero-elements (*N*, *S*) may be taken as a measure of contamination by non-related substances (i.e.: solvents, salts, etc.) as long as these contaminants do not contain the hetero-element. A comparison with ^{1}H NMR data was accomplished [25] for 98 different compounds with a range of strength extending from 80% to 100% (^{1}H NMR value). The results are shown in Fig. 2. Two groups of data were excluded from the correlation analysis: seven compounds with ^{1}H NMR evidence of nitrogen containing impurities (black triangles in the figure), two compounds where insufficient signal resolution between impurities and the test molecule prevented accurate assessment of purity in the ^{1}H NMR spectrum (empty triangles in the figure). For all other compounds (89), a correlation coefficient $r^2 = 0.907$ and a nearly unitary straight-line slope (0.95) were obtained. These figures may appear crude, but the limited value of elemental analysis as a criterion of purity should not be overlooked. Actually, the application of quantitative ^{1}H NMR offers a novel opportunity to organic chemistry, since the determination of quantitative purity, nowadays applied only to a few, well-characterized compounds, may well become routine.

7. Absolute purity (strength) of bulk chemicals and analysis of mixtures

Application of quantitative NMR is not limited to highly pure compounds; the intrinsic specificity obtained at high fields makes qNMR suitable to the analysis of mixtures. There are limitations, however, when signal overlapping precludes identification and integration of the target molecule resonances. The results of Fig. 2 highlight the success rate of ^{1}H NMR method in a typical environment of medicinal chemistry laboratory: signal overlap prevents a reliable determination of purity only in two out of 98 cases. Nevertheless, the difference with elemental analysis amounts to just a few percent. More common is the case of nitrogen containing impurities (e.g.: dimethylformamide, ammonium salts), where the N_{found}/N_{calc} ratio is totally misleading at measuring purity.

With chemical libraries, the method fails more frequently (∼11% of the samples in the reported investigation were not suitable for qNMR analysis [13]). These samples, however, are usually the poorest in purity and should be discarded if the quality of a library has to be maintained at a high level.

For 53 compounds with various levels of purification, including some synthetic intermediates and raw materials, qNMR obtained values of strength were compared [25] with those determined according to guidelines for pharmaceutical products [23]: strength = 100 − (impurities − solvents − water − residue on ignition). A very good correspondence was found in the high purity range (strength > 90%), whereas a tendency at overestimating strength with the traditional approach was apparent for the low purity compounds (strength < 90%). This result has been interpreted as an indication of better accuracy for the qNMR approach,

which is not affected by the approximations contained in the above equation.

One case where qNMR has no rival is the determination of true reaction yields of compounds synthesized for the first time. In our experience, even with crude products (quantitative purity ∼50%) the estimate is generally possible, on the basis of one or more well-resolved NMR resonance. This feature is particularly convenient at determining yields in the preparation of chemical libraries [33,34,39].

8. Future opportunities: stability and impurities

The high accuracy and precision of the NMR method makes it suitable for the evaluation of chemical stability, though this is not an explored area yet. As usual, the main advantage with respect to chromatography is expected in method development, since NMR would generally require only sample dissolution, whereas signal separation will be in most cases warranted. This characteristic makes the NMR method best suitable for the R&D environment, where many different compounds are analyzed and method development by HPLC is a large fraction of the total operator involvement. In a QC environment, where methods are well settled, the advantage is probably less significant. Nevertheless, even in such a regulated environment, NMR may be convenient for compounds lacking of suitable chromophores for LC-UV analysis. Recently, Deubner and Holzgrabe [40] reported a comparative investigation of the purity and composition of some aminoglycoside antibiotics, which lack of a proper UV chromophore. Of the three applied, orthogonal techniques (HPLC, NMR and Micellar Electrokinetic Chromatography, MEKC) NMR and MEKC gave consistent and satisfactory results, with a distinctive advantage towards the less robust HPLC.

Henderson [30] has shown that, for impurities, a 0.1% detection limit is achieved with solutions containing about 26 mg/mL of the main component. A similar evaluation is reported by Maniara et al. [18] and by Wells et al. [22]. These encouraging results open up interesting opportunities for the detection and quantification of impurities via qNMR.

9. Conclusions

Quantitative NMR is now established as a rapid and generic method for determining concentration, purity, reaction yield, and mixture composition. When combined with the high level of information delivered on molecular structure, the two features make NMR a complete and unique method for the qualitative and quantitative characterization of new synthetic molecules. Even for small chemical libraries (100–200 compounds), the burden of spectra interpretation is tolerated if NMR is regularly used during the library development. For example, in a recent investigation on benzimidazole derivatives [39], Vourloumis et al. positively comment the use of qNMR in the characterization of chemical libraries, since this technique adds *"enormous flexibility to solid phase organic synthesis in terms of simultaneous quality and quantity control of the produced compounds"*.

On the other hand, the great precision and accuracy which are obtained with qNMR make this an ideal reference method for the evaluation of other quantitative tools [4]. A more thorough and widespread application of this technique than is currently accepted is highly desirable.

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