This is the first of a series of articles related to the launch of new organic CRMs certified by quantitative NMR under double accreditation. In this issue, we focus on CRMs intended for use as internal standards in qNMR, while in the next issue of Analytix we will present a first series of certified organic standards for chromatography.

The quantification of content or purity of organic substances has up to now often been performed with chromatographic techniques such as HPLC or GC. Over the last years, quantitative NMR (qNMR) has evolved not only in the pharmaceutical industry but also in many other fields.

In the R&D lab of Sigma-Aldrich® Buchs (Switzerland) a new Bruker Avance III 600 MHz NMR spectrometer was installed recently for high-resolution qNMR measurements of organic substances. By the end of 2009, the new NMR lab was fully accredited under both ISO/IEC 17025 [1] and also ISO Guide 34 [2] for the certification of organic reference materials using 1H-qNMR. The combination of both accreditations represents the highest achievable reliability level and earns the label of “gold standard” among CRM producers.

A set of calibration standards for use as internal standards in qNMR is being launched as a first series of new CRMs. These CRMs are comprehensively characterised by qNMR and other techniques, leading to the highest accuracy and very low uncertainties of the certified values. All of these qNMR reference materials are traceable to internationally accepted references from NIST and a detailed certificate is delivered with each CRM.

Performance and advantages of qNMR measurements

Quantitative NMR shows many advantages over other analytical techniques with regard to quantification or purity determination of organic substances. The most outstanding attribute of qNMR is that it is a relative primary method: the signal intensity is in direct proportionality with the number of protons contributing to the resonance. The structures of the chemical substances are therefore irrelevant. In addition, no significant empirical factors or unknown biases contribute to the ratio of signals. The signal ratio of two different protons can therefore be measured with tremendous precision and the only significant contribution to the measurement uncertainty is the integration of the signals. In other words, the direct response of a qNMR experiment is of the greatest accuracy.

Of course, a few basic requirements must be met in order to obtain good qNMR results. To begin with, the weighing of the reference and the sample prior to the qNMR measurements must be done with utmost accuracy, since this is the essential sample preparation step in qNMR analysis. It is self-evident that the availability of a suitable reference material for internal calibration is mandatory (see next section). Furthermore, the reference substance and the sample must not react with each other or with the solvent. The relevant signals which are selected for the measurement must be clearly separated from each other and also from other signals. Appropriate instrument settings are required so that no intensity is lost through incomplete relaxation.

Requirements to qNMR calibration standards
Since NMR signal intensity is fully independent of the nature of the substance, many organic substances could potentially serve as an internal reference for qNMR. In reality, the number of suitable candidates for qNMR references is rather limited, since they must conform to a series of the following specific requirements: only substances with a very limited number of signals and of highest organic purity are suitable as a qNMR reference. The candidates must be stable in solution and should not be chemically reactive. They should not contain residual water, since the presence of water can lead to line broadening or baseline distortion in many cases. Of course, to ensure proper weighing results, a solid qNMR reference should not be hygroscopic and a liquid qNMR reference should not be volatile. The number of isochronic proton nuclei be optimum, that is, neither too high nor too low in relation to the molecular mass of the qNMR reference substance, since a 1:1 signal intensity ratio of sample and internal standard is targeted. Otherwise, the amount of substance needed for one measurement would be either very low (leading to bad weighing result) or too high (causing solubility problems). The signals of a qNMR reference should not cover areas in the spectrum where most often the analyte signals are expected to be. Finally, a qNMR reference substance should be neither extremely toxic nor carcinogenic or mutagenic.

It is obvious that many different qNMR standards must be available to cover the broad variety of analytes and deuterated solvents. We have developed a series of qNMR reference substances whereby every reference substance has its individual solubility behaviour and chemical shift. The selection of new qNMR calibration standards is given in Figure 1 (water soluble standards) and Figure 2 (standards soluble in apolar organic solvents).
Figure 1 $^1$H NMR spectra of water soluble qNMR standards. The residual signal of HDO is partly suppressed
Certification of calibration standards for qNMR

To achieve all the above-mentioned requirements, the new class of certified qNMR reference substances undergo a series of pre-tests and extensive certification measurements in order to provide compliance with ISO/IEC 17025 and ISO Guide 34 requirements. Only high-purity materials are carefully sourced or purified and extensively characterised by chromatography, LC-MS and other standard techniques before they undergo the qNMR certification procedure.

- **Pre-testing:** the substance is tested for hygroscopy or volatility, stability in different deuterated solvents, and reactivity against NIST certified reference substances (>24 hour period).
- **Stability testing:** Replicate tests after storage at higher temperature are performed to ensure that even extreme transportation conditions do not affect the certified value (>1 month period). Data on ageing studies at the recommended storage temperature are used to define the shelf life of the products (>3 year period).
- **Signal suitability test:** Since only “pure” signals are allowed for NMR quantification (no overlapping and no underlying impurity signals), extensive 2D experiments are required (H-H COSY and C-H correlated experiments in some cases). With H-H COSY experiments,
hidden signals from impurities can easily be detected with a limit of detection below 0.1% (see example in Figure 3). This suitability check is performed before the certification measurements are started.

- Homogeneity and traceability: 10 samples are taken from different places within the batch. The NMR samples are then prepared by weighing equivalents of the qNMR substance and the NIST reference together in one vial, using an ultramicro balance. The samples are dissolved, measured and analysed further under standardised measurement settings on a Bruker Avance III 600 MHz NMR instrument. Following the analysis of variances, a detailed uncertainty budget is calculated and all the data is summarised in a comprehensive certificate.

![Figure 3](image)

**Figure 3** 2D COSY of 3,5-dinitrobenzoic acid: the cross peaks of the impurity 3-nitrobenzoic acid can be identified although the relative content of this impurity is below 0.1%.

By this quality procedure, most qNMR reference substances are obtained with purities ≥ 99.5% and have typical uncertainties ≤ 0.2%. All qNMR standards are packed in brown-glass bottles and closed with a sealed cap. A detailed certificate in accordance with ISO Guide 31 is delivered with each CRM. The certificate contains a comprehensive documentation tabulating lot-specific values, traceability, uncertainty calculation, expiration date and storage information.

Since qNMR offers several advantages over other analytical techniques, Sigma-Aldrich® will launch a new series of certified organic standards for chromatography whereby the certified value of these CRM will not be measured by HPLC or GC only but also by qNMR.
References

1. ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories.
2. ISO Guide 34, General requirements for the competence of reference material producers.