QUANTITATIVE NMR

Technical Details and *Trace*CERT® Certified Reference Materials

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Quantitative NMR is increasingly used in Pharmaceutical and Chemical Industry as an efficient tool to quantify organic molecules. Most commonly, proton NMR is applied. However, the implementation of qNMR in new fields of application (e.g. metabolomics, biomarker discovery, physiological pathways) brings along more complex molecules and systems, thus making the usage of ^{1}H-qNMR challenging. The use of other NMR active nuclei, namely ^{31}P or ^{19}F can be a better option in such cases. In this brief brochure, we would like to introduce you to the exciting analytical technique of quantitative nuclear magnetic resonance spectrometry (qNMR).

In 2009, Sigma-Aldrich Switzerland started to apply qNMR under ISO/IEC 17025 and ISO Guide 34 accreditation to manufacture organic certified reference materials (CRMs). Using a set of more than 20 different internal qNMR standards for ^{1}H, ^{13}P and ^{19}F nuclei we built up a considerable portfolio of CRMs for chromatography. So far, more than 200 products are available including pesticides, polyaromatic hydrocarbons (PAH), phenols, plasticizers, cosmetics, antibiotics, air monitoring substances, amino acids, organic pollutants, natural substances and fatty acids. We continuously refined and optimized our qNMR skills, also taking into account valuable input from CRM users.

In the following, we will share some details that will help you to use your NMR instrument for quantitative measurements, achieving maximum accuracy and reproducibility. Moreover, we will present the *Trace*CERT CRMs, available from Sigma-Aldrich, which we use to certify organic CRMs and which will enable you to get reliable results traceable to a primary standard of an NMI (eg NIST or NMIJ) and hence traceable to SI.

Quantification of Organic Compounds

Since most analytical techniques are compound dependent, reliable quantification of organic material is a very challenging task. For example, using HPLC with UV, DAD or fluorescence detection always requires a traceable reference of the very same compound. However, for most organic compounds, no reliable reference material is available.

Therefore, the content of an organic material is usually determined by measuring all potential impurities (such as related compounds, water, residual solvents and inorganic impurities) and calculating the content by subtracting the impurity values from a total of 100%. This method however implies that no potential impurities have been overlooked and that related impurities measured by a chromatographic method have the same response as the target analyte, which is often not the case.

An alternative to this laborious method is using a relative primary method, such as qNMR. While NMR has been one of the most important qualitative methods for structure elucidation of organic compounds for the past 40 years, its quantitative use has gained increasing importance over the past decade.[1]

Using Internal or External Calibration

Different referencing techniques have been tested for qNMR, internal as well as external. Bharti and Roy gave a broad overview over various methods including pros and cons.[2]

External referencing approaches comprise NMR-tubes with coaxial inserts leading to a separation of analyte and standard. Furthermore, electronic reference methods have been elaborated, e.g. ERETIC (Electronic REference To access In vivo Concentration), using an electronically generated signal as the internal reference signal. Since the achievement of low measurement uncertainties is a key issue for the development of CRM, several authors described the use of the internal reference method.[3,4,5]

We usually prefer the internal reference method (Figure 1) although the external standard method also certainly has its advantages, e.g., easier recovery of the analyte material, which may be of importance if very expensive material is analyzed. However, with the internal standard method, much higher precision and lower uncertainties can be achieved. Once the materials have been weighed into the same vial, the ratio of analyte and reference stays the same. In contrast to the external standard method, the amount of added solvent, and hence the concentration of the solution, is not critical for the quantification calculation.

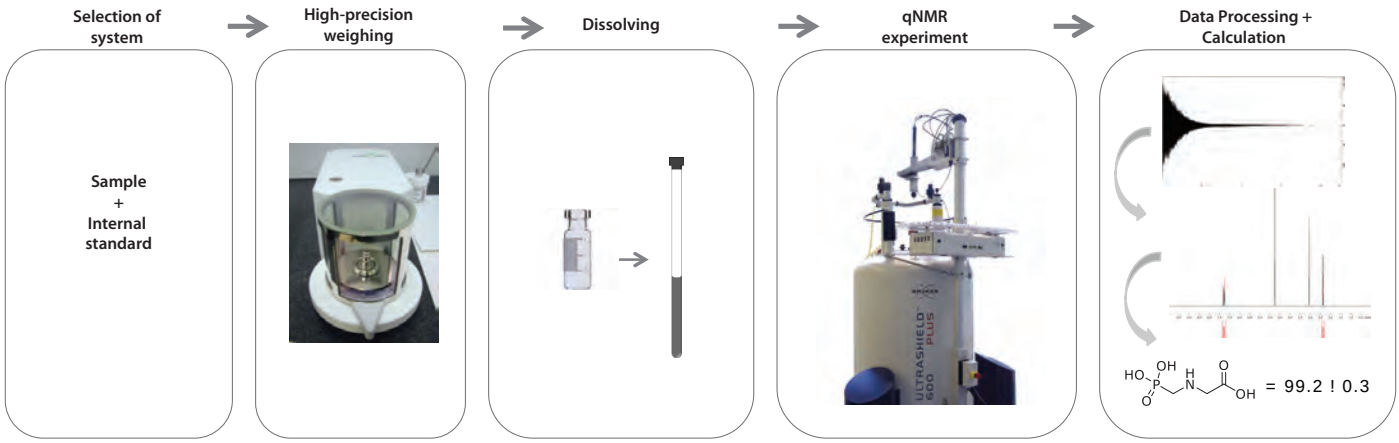


Figure 1: General procedure of a qNMR experiment using an internal standard

Things to Consider Step-by-Step

The following recommendations refer to the internal standard method, although most of them are equally relevant for the external standard method.

1. Selection of a Suitable Reference Material

QNMR reference materials should be:

Highly pure	→	Minimizes overlap
Non-hygroscopic and non-volatile	→	Ensures stability and eases weighing
Free of residual water	→	Minimizes baseline effects
Highly soluble in various common deuterated NMR solvents	→	Provides unique and stable signals and chemical shift
Preferably have only a few signals	→	Minimizes overlap with analyte signals

Different samples require different references, but it is sufficient to have one well separated signal from each compound in the spectrum. In order to generate accurate and precise qNMR measurements, a signal intensity ratio of 1:1 is recommended, but not mandatory.

All commercially available qNMR *Trace*CERT materials (see page 5) fulfill the required properties, are traceable to NIST SRMs or NMIJ SRMs and can directly be employed as CRMs in qNMR analyses.

2. Compatibility Checks

After having selected a suitable reference standard, especially in the case of internal calibration described here, the mixture must be checked to determine its chemical stability and inertness in order to avoid reactions between sample and reference or solvent. We recommend compatibility checks with NMR experiments, measuring the tolerance between sample and standard in the mixture by running experiments at t=0 and at a second time, which includes the expected experimental time for all planned repetitions, e.g., t=5, 12 or 24h. The relevant signals for quantification must not overlap with each other nor interfere with possible impurities. It is often necessary to try different combinations of solvent and internal standard in order to find the most favorable analytical setup.

3. Metrological Weighing

Reliable weighing values are mandatory, as they have direct influence on the result, and are recommended to be performed in a metrological way. Using a micro-balance or a similar one is key to success. Using a less sensitive balance will lead to higher uncertainty contributions by the weighing procedure, but this is a question of what level of precision should finally be achieved. We use a XP6U Ultra-microbalance from METTLER TOLEDO with a readability of 100 ng, certified by DKD and calibrated with OIML Class E2 weights. The balance is positioned on a 700 kg stone table, inside a safety weighing cabinet and a U-electrode is in place to get rid of potential static charge. This arrangement also reduces potential air fluctuations caused by the lab door, air conditioning or heating. Climate conditions are tracked in parallel to each weighing step, allowing subsequent air buoyancy correction. Sample and reference are not weighed into the NMR-tube directly, but rather into an HPLC-vial, which can be resealed for solvation after having added the deuterated solvent. Try to avoid obvious weighing errors, e.g., eccentric load, and use glass vials and metal spatula, not plastic, due to possible static charge.

4. Instrument Settings

We are working with a Bruker Avance III 600MHz spectrometer equipped with 5 mm BBO probe head and a Bruker Avance III 600MHz spectrometer equipped with a 5-mm CPP TCI probe head. Instruments with a higher or lower magnetic field are suitable for quantification as well. If high precision should be achieved and if time is not an issue, it is recommended to apply a 90° pulse instead of a 30° pulse to improve the signal-to-noise-ratio (S/N) and reduce artifacts. No spinning is applied in order to avoid spinning side bands that could complicate the spectrum.

In any case, T_1 relaxation time has to be determined in the mixture before the actual qNMR experiment is started. This is the most critical item for consistent results. T_1 relaxation time can be measured using the inversion recovery experiment operating on the basis of a 180° pulse, followed by a 90° pulse after a variable delay. **Table 1** lists T_1 relaxation times for *Trace***CERT®** CRMs in different deuterated solvents. Since T_1 relaxation times vary with the concentration, the mixture and the solvent we recommend evaluating the relaxation time in the mixture, e.g., simultaneously with the compatibility check. In order to calculate D_1, the longest resulting T_1 (relaxation delay) in a mixture has to be multiplied by a minimum factor of 7.

5. Spectra Evaluation

After zero filling and exponential weighing of the FID, the Fourier Transformation is applied. Phase- and baseline correction are two critical steps in processing the spectra. In order to obtain accurate integrals, it is preferable to perform this process manually instead of relying on automatic procedures. It is important to always perform the integration step in the same way for both signals, which means either include the ^{13}C-satellites or exclude them for either signal. The decision depends very much on signal shape and potential impurities near the signals of interest.

6. Content Calculation

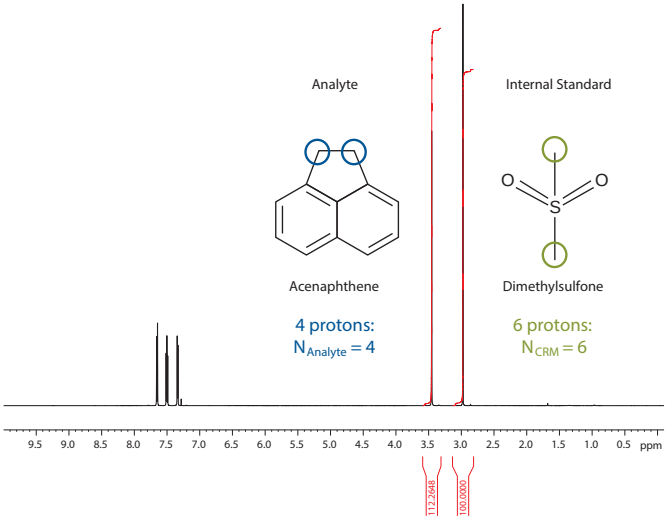


Figure 2: Example of a ^{1}H qNMR Spectrum with internal standard calibration.

We recommend performing 5–10 replicates in order to get a relevant statistical contribution to the overall measurement uncertainty. Quantification of the sample content is directly calculated from the NMR peak integrals, together with the initial weights of sample and reference substance, molecular masses, number of protons contributing to the respective signals and the certified purity of the reference standard. Please note that any sample contains the analyte plus potential impurities, leading to a slight distinction which is indicated by different subscripts within the formula.

$$P_{Sample} = \frac{I_{Analyte}}{I_{CRM}} \cdot \frac{N_{CRM}}{N_{Analyte}} \cdot \frac{M_{Analyte}}{M_{CRM}} \cdot \frac{m_{CRM}}{m_{Sample}} \cdot P_{CRM}$$

P_{Sample}	Purity of the sample as mass fraction
P_{CRM}	Purity of the CRM as mass fraction
$I_{Analyte}$	Integral of the analyte signal
I_{CRM}	Integral of the CRM signal
$N_{Analyte}$	Number of analyte protons (phosphorus nuclei, fluorine nuclei)
N_{CRM}	Number of CRM protons (phosphorus nuclei, fluorine nuclei)
$M_{Analyte}$	Molecular mass of the analyte
M_{CRM}	Molecular mass of the CRM
m_{Sample}	Mass of sample
m_{CRM}	Mass of CRM

In order to obtain even more precise results, weighing values can be corrected for air buoyancy if climate conditions in the lab and also the densities of the materials are known.

7. Uncertainty Budget

In addition to the statistical contribution (Type A), there are also a couple of other contributions (Type B). All parameters in the equation mentioned above contribute to the overall measurement uncertainty budget, but with different magnitude.[6] It is zero for the number of protons in small molecules, it is very small for the molecular masses and the weighing values (dependent on the balance used) and it is medium for the individual contribution of the operator. The biggest contributions may come from the repeatability or from the purity of the reference standard. In addition to these uncertainty contributions for the qNMR measurement, the homogeneity and stability of the material have to be assessed (although this is only relevant for a CRM producer), resulting in analysis of variance (ANOVA) data that may be included in the calculation of uncertainty.[7] The recommended minimal sample size is stated in the certificate, as well as a hard expiry date. In most cases, the measurement uncertainty is enhanced by a coverage factor of k=2.

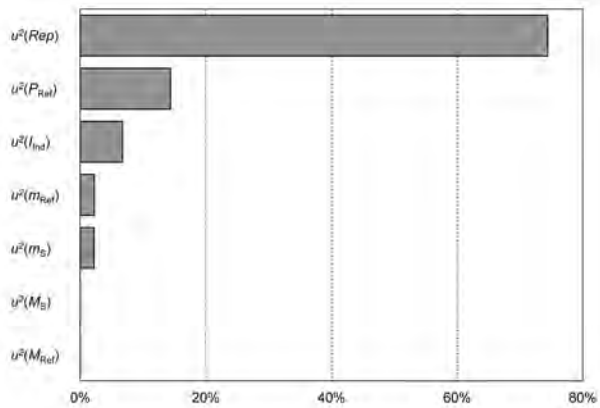


Figure 3: Overview on typical contributions to the relative standard uncertainty (relative squared contributions are given) for the quantification of tris(2-chloroethyl) phosphate using phosphonoacetic acid as internal standard in a ^{31}P qNMR measurement.

Thus, following some basic rules, and with a good balance, it is possible to routinely perform qNMR measurements with 0.5 - 1% measurement uncertainty. In order to minimize the increments by the internal reference, Sigma-Aldrich certified its *Trace*CERT CRMs under optimal conditions, so that their uncertainties down to 0.1% would not dominate the overall result.[3]

Standards for ^{1}H quantitative NMR, TraceCERT®

		D_2O			$CDCl_3$			DMSO-d_6			CD_3OD			CD_3CN		
PN	Substance	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)
01380	Ethylene carbonate	4.5	5.5	>250	4.5	7.0	>250	4.5	2.7	>250	4.5	5.3	>250	4.5	2.0	>250
03826	Calcium formate	8.4	3.4	>250	-	-	-	-	-	-	-	-	-	-	-	-
06185	Benzoic acid	-	-	-	8.1 7.7 7.5	3.7 4.0 3.4	150	8.0 7.6 7.5	3.3 3.7 3.0	>250	8.0 7.6 7.5	4.3 4.4 3.9	>250	8.0 7.7 7.5	4.5 2.5 2.6	80
06856	Duroquinone	-	-	-	2.0	3.3	>250	2.0	3.1	30	2.0	4.0	10	2.0	5.4	20
07038	Dimethyl terephthalate	-	-	-	8.1 4.0	3.6 1.8	160	8.1 3.9	2.9 1.1	20	8.1 4.0	4.4 2.4	4.0	8.1 3.9	4.9 2.6	20
14659	Potassium hydrogen phthalate	7.5	2.5	>250*	-	-	-	-	-	-	7.9	2.5	5.0	-	-	-
40384	1,2,4,5-Tetrachloro-3-nitro-benzene	-	-	-	7.8	10.7	>250	8.5	12.6	>250	9.4	9.6	50	6.8	6.4	10
41867	Dimethyl sulfone	3.0	2.9	>250	3.0	2.7	80	3.0	2.4	>250	3.0	3.3	40	2.9	2.6	>250
42582	Ethyl 4 (dimethyl-amino) benzoate	-	-	-	7.9 6.7 4.3 3.1 1.4	3.8 2.4 2.8 2.0 2.5	>250	7.8 6.7 4.2 3.0 1.3	2.5 1.4 1.9 1.5 2.1	>250	7.9 6.7 4.3 3.0 1.4	3.4 2.5 3.3 2.2 2.7	>250	7.9 6.7 4.3 3.0 1.3	5.6 3.7 4.1 3.6 3.6	50
50409	Thymol	-	-	-	7.1 6.8 6.6 3.2 2.3 1.3	3.8 4.5 4.8 4.3 3.1 1.9	>250	7.0 6.7 6.5 3.1 2.2 1.1	2.0 2.2 2.6 2.3 2.0 0.9	>250	7.0 6.6 6.5 3.2 2.2 1.2	3.7 4.4 5.8 4.0 2.8 1.8	>250	7.1 6.7 6.6 3.2 2.2 1.2	4.9 5.7 5.7 5.2 3.5 2.4	>250
55177	Benzyl benzoate	-	-	-	5.4 8.1	4.3 13.6	>250	5.4 8.0	2.2 8.8	>250	5.4 8.1	2.0 3.9	>250	5.4 8.1	1.0 2.4	>250
74599	1,3,5-Trimethoxy-benzene	-	-	-	6.1 3.8	4.7 2.2	>250	6.1 3.7	3.2 1.4	>250	6.1 3.8	4.8 2.7	>250	6.7 4.3	3.4 2.6	>250
74658	1,2,4,5-Tetramethyl-benzene	-	-	-	7.0 2.2	6.1 4.0	>250	6.9 2.1	4.7 2.6	10	6.9 2.2	5.9 4.3	10	6.9 2.2	7.7 5.0	50
89151	Dimethylmalonic acid	1.3	1.0	100	-	-	-	1.3	0.7	250	1.4	1.0	250	1.4	2.0	30
92816	Maleic acid	6.3	6.1	>250	-	-	-	6.3	3.0	>250	6.3	3.9	10	6.4	2.3	20
94681	Methyl 3,5-dinitrobenzoate	-	-	-	9.3 9.2 4.1	8.0 6.1 2.6	>250	9.1 8.9 4.0	9.4 7.6 1.5	100	-	-	-	9.1 9.0 4.0	9.0 8.2 3.5	>250

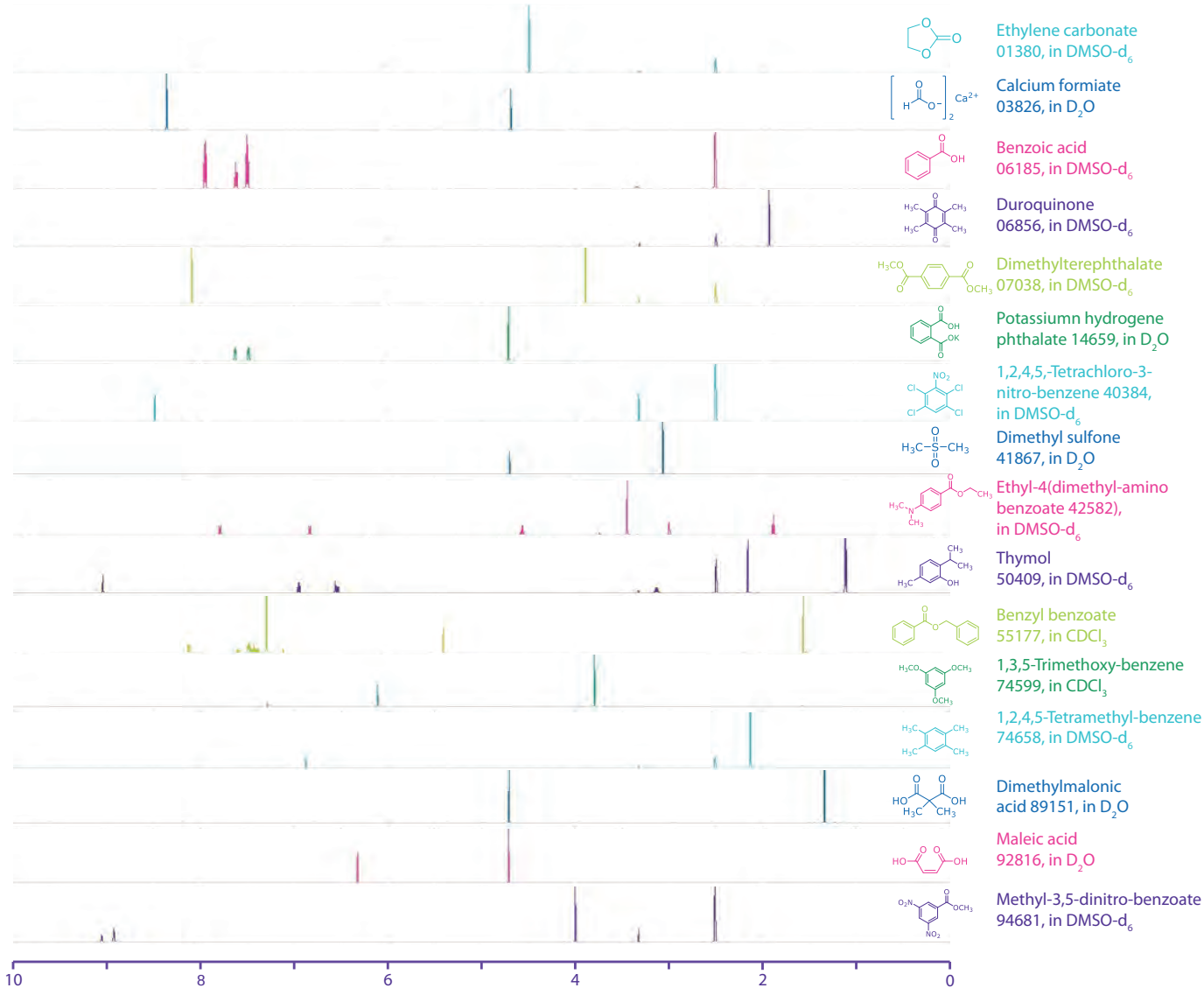
Standards for ^{31}P quantitative NMR, TraceCERT®

		D_2O			$CDCl_3$			DMSO-d_6			CD_3OD			CD_3CN		
PN	Substance	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)
05498	Triphenyl phosphate	-	-	-	-17.7	2.7	>250	-17.3	1.2	>100	-17.5	3.1	>10	-17	4.3	>250
92214	Potassium phosphate monobasic	0.08	8.0	>250	-	-	-	-	-	-	-	-	-	-	-	-
96708	Phosphonoacetic acid	15.7	4.6	>250	-	-	-	14.9	1.5	>250	17.7	2.9	>250	-	-	-

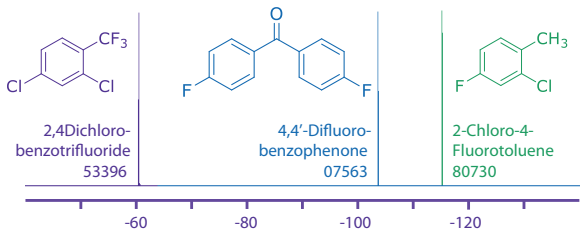
Standards for ^{19}F quantitative NMR, TraceCERT®

		D_2O			$CDCl_3$			DMSO-d_6			CD_3OD			CD_3CN		
PN	Substance	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)	δ (ppm)	T1 (s)	s (mg/ml)
07563	4,4'-Difluoro-benzophenone	-	-	-	-105.8	2.4	>250	-106.5	1.4	150	-108.1	2.8	30	-108.3	2.3	140
53396	2,4-Dichloro-benzotrifluoride	-	-	-	-62.5	2.3	>250	-61.2	1.2	>250	-65.4	3.3	>250	-63	2.9	>250
80730	2-Chloro-4-fluorotoluene	-	-	-	-115.8	4.4	>250	-115.3	3.3	>250	-117.7	4.8	>250	-117.3	4.7	>250

Standards for ^{1}H quantitative NMR, TraceCERT®



Standards for ^{19}F quantitative NMR, TraceCERT®



Standards for ^{31}P quantitative NMR, TraceCERT®

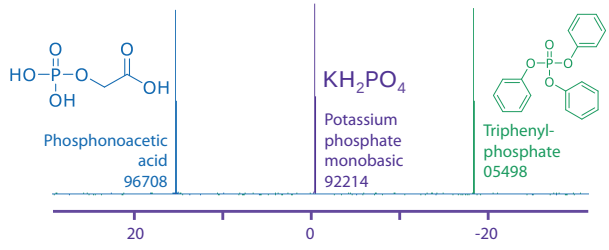
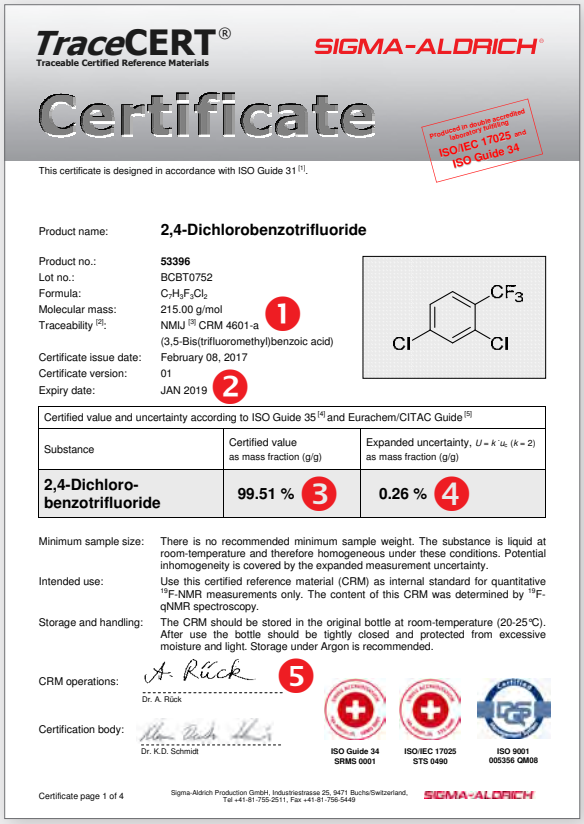


Figure 4: Example spectra for all *Trace***CERT** CRMs for qNMR in organic solution and / or D_2O respectively



TraceCERT®
Traceable Certified Reference Materials

SIGMA-ALDRICH

Certificate

Produced in double accredited laboratory holding ISO/IEC 17025 and ISO Guide 34

This certificate is designed in accordance with ISO Guide 31 [1].

Product name: **2,4-Dichlorobenzotrifluoride**

Product no.: **53396**
Lot no.: BCBT0752
Formula: $C_7H_3F_3Cl_2$
Molecular mass: 215.00 g/mol
Traceability [2]: NMIJ [3] CRM 4601-a (3,5-Bis(trifluoromethyl)benzoic acid)
Certificate issue date: February 08, 2017
Certificate version: 01
Expiry date: JAN 2019

1 2

Certified value and uncertainty according to ISO Guide 35 [4] and Eurachem/CITAC Guide [5]

Substance	Certified value as mass fraction (g/g)	Expanded uncertainty, $U = k \cdot u_c$ ($k = 2$) as mass fraction (g/g)
2,4-Dichloro-benzotrifluoride	**99.51 %** 3	**0.26 %** 4

Minimum sample size: There is no recommended minimum sample weight. The substance is liquid at room-temperature and therefore homogeneous under these conditions. Potential inhomogeneity is covered by the expanded measurement uncertainty.

Intended use: Use this certified reference material (CRM) as internal standard for quantitative ^{19}F-NMR measurements only. The content of this CRM was determined by ^{19}F-qNMR spectroscopy.

Storage and handling: The CRM should be stored in the original bottle at room-temperature (20-25°C). After use the bottle should be tightly closed and protected from excessive moisture and light. Storage under Argon is recommended.

CRM operations: Dr. A. Rück 5

Certification body: Dr. K.D. Schmidt

ISO Guide 34 SRMS 0001 | ISO/IEC 17025 STS 0490 | ISO 9001 005356 QM08

Certificate page 1 of 4

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SIGMA-ALDRICH

1. Traceability Statement
2. Expiration Date
3. Certified Value (g/g)
4. Expanded Uncertainty (g/g)
5. Signatures and Accreditation Stamps

Figure 5: Example of a Certificate 94681 Methyl 3,5-dinitrobenzoate (first page)

*Trace*CERT Certified Reference Materials for qNMR

We are currently providing a series of 16 diferent certified refernce materials (CRMs) designed for use in ^{1}H qNMR experiments and each 3 different CRMs for the use in ^{19}F or ^{31}P qNMR experiments. All products are either traceable to NIST (National Institute of Standards and Technology) SRM or NMIJ (National Metrology Institute of Japan) SRM and are produced under ISO/IEC 17025 and ISO Guide 34 double accreditation, which corresponds to the highest achievable quality level and is also referred to as the "gold standard".

In **Table 1** on page 5, the 22 CRMs are listed, including chemical shifts of the signals, values for solubilities and relaxation times. In combination with the example spectra shown in **Figure 4**, this should help you to identify the best suitable reference material for your qNMR task.

Finally, **Figure 5** shows the first page of an example certificate, highlighting the most important features.

We continuously update our product range.
The current product list can be found at
sigma-aldrich.com/qnmr

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