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# Survey and qualification of internal standards for quantification by $^1$H NMR spectroscopy

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ABSTRACT

In quantitative NMR (qNMR) selection of an appropriate internal standard proves to be crucial. In this study, 25 candidate compounds considered to be potent internal standards were investigated with respect to the ability of providing unique signal chemical shifts, purity, solubility, and ease of use. The $^1$H chemical shift ($\delta$) values, assignments, multiplicities and number of protons (for each signal), appropriateness (as to be used as internal standards) in four different deuterated solvents ($D_2O$, DMSO-$d_6$, $CD_3OD$, $CDCl_3$) were studied. Taking into account the properties of these 25 internal standards, the most versatile eight compounds (2,4,6-triiodophenol, 1,3,5-trichloro-2-nitrobenzene, 3,4,5-trichloropyridine, dimethyl terephthalate, 1,4-dinitrobenzene, 2,3,5-triiodobenzoic acid, maleic acid and fumaric acid) were qualified using both differential scanning calorimetry (DSC) and NMR spectroscopy employing highly pure acetanilide as the reference standard. The data from these two methods were compared as well as utilized in the quality assessment of the compounds as internal standards. Finally, the selected internal standards were tested and evaluated in a real case of quantitative NMR analysis of a paracetamol pharmaceutical product.



## 1. Introduction

Quantitative NMR (qNMR) was introduced already more than 50 years ago and the first study on pharmaceuticals was published in 1963 [1,2]. The state of the art of qNMR in the field of pharmaceuticals and related areas was reviewed [3–6] and during the recent years scientific publications emerged at an escalating rate.

An advantage of qNMR compared to other instrumental analytical methods is that it is a primary ratio method of measurement, since the peak areas are proportional to the number of corresponding nuclei giving rise to the signals [7–9]. The uncertainty in quantification measurement by NMR spectroscopy is low [10,11] and the performance of qNMR is similar to that of the alternative analytical techniques [12]. Compared to the traditional chromatographic methods that are still favored in routine quantitative analyses, NMR spectroscopy have certain advantages [10] such as (i) simple and easy sample preparation, (ii) possibility to simultaneously determine molecular structures, (iii) no need for individual experimental setup, *e.g.*, reference of the same compound and calibrations, (iv) relatively short measuring times, (v) non-invasive and non-destructive character of the method, (vi) no need for prior isolation of the analyte present in a mixture, (vii) possibility of simultaneous quantitative analysis of multiple target analytes in a mixture.

The applicability of qNMR has increased due to availability of high sensitivity/homogeneity NMR systems together with modern software packages which allow accurate and precise data processing. In qNMR a reference standard of the analyte is not needed, *i.e.*, the quantification may be performed using an internal standard [1,5]. Various internal standards have been used in qNMR [6], usually co-dissolved with the analyte, but also introduced in a separate coaxial insert tube [13]. In recent time also quantification towards an electronically generated signal (peak) in the NMR spectrum, so-called Eretic, was evolved [14]. However, a descriptive and comparative study on internal standards is non-existent in the literature.

The scope of the present work was to study compounds that can be used as internal standards using $^1$H NMR experiments, since $^1$H nuclei are at the core of qNMR applications due to high sensitivity

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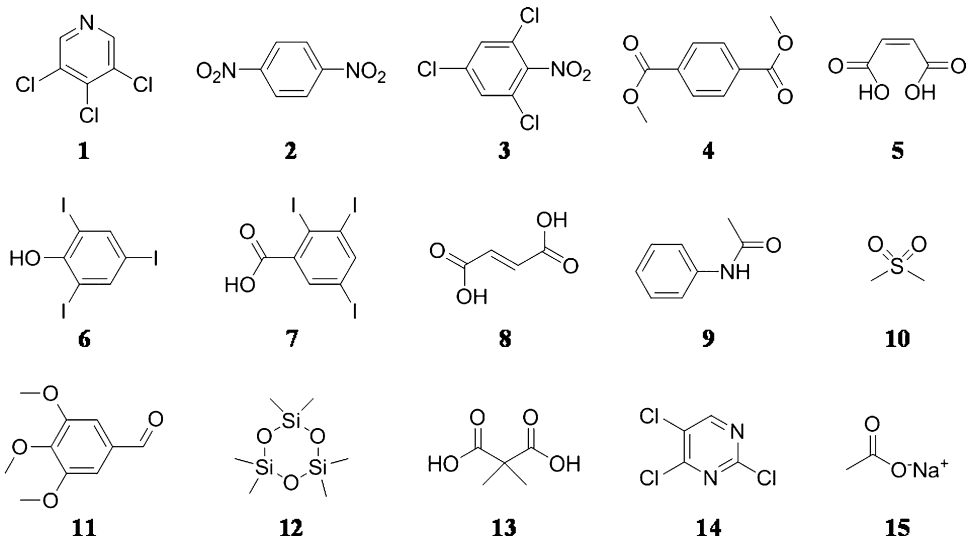


**Chart 1.**

and widespread presence in organic molecules, even though other NMR active nuclei like $^{13}C$, $^{19}F$, and $^{31}P$ can be employed [1]. In qNMR the signal from the internal standard and the signal from the analyte must not overlap, and in the selection of compounds in this study attention was attempted so as to avoid normally crowded spectral regions.

In the present work 25 candidate qNMR internal standards were thoroughly investigated. Chemical shift ($\delta$) values, assignments, multiplicities and number of protons (for each signal), appropriateness (as to be used as internal standards) of 25 compounds in four different deuterated solvents ($D_2O$, DMSO-$d_6$, $CD_3OD$, $CDCl_3$) were examined. Some of these compounds were previously used in NMR quantification, while some others are novel. A method for qualification of the standards was also developed, in order to ensure accurate and precise qNMR measurements. Quantification of a pharmaceutical product was also performed using selected internal standards.

## 2. Experimental

### 2.1. Materials and chemicals

Internal standard substances, whose abbreviations and certified purities from the manufacturer are given in respective parentheses, were as follows: dimethyl terephthalate (**4**, 100.0%), maleic acid (**5**, 100.0%), fumaric acid (**8**, 100.0%), acetanilide (**9**, *N*-phenylacetamide, 99.99%), dimethyl sulfone (**10**, 99.9%), 3,4,5-trimethoxybenzaldehyde (**11**, 99.4%), hexamethylcyclotrisiloxane (**12**, 99.9%), dimethylmalonic acid (**13**, 99.8%), 5-fluorouracil (99.9%), anthracene (99.0%), acetamide (99.9%) and L-leucine (100.0%) were purchased from Sigma Aldrich, Stockholm, Sweden, 3,4,5-trichloropyridine (**1**, 99.9%), 1,3,5-trichloro-2-nitrobenzene (**3**, 98.65%), 2,4,6-triiodophenol (**6**, 97.8%), 2,3,5-triiodobenzoic acid (**7**, 99.3%), 2,4,5-trichloropyrimidine (**14**, 99.4%), 2,4,6-trifluorobenzoic acid (99.8%), 2,3,4-trifluorobenzamide (99.9%) and 2,4,6-trichlorobenzoyl chloride (99.0%) were purchased from Fisher Scientific, Göteborg, Sweden, 1,4-dinitrobenzene (**2**, 99.9%) and 2,3,5-tribromothiophene (98.2%) were purchased from Alfa Aesar, Frölunda, Sweden, and sodium acetate (**15**, >99%), hexamine (99.5%), uracil (99.8%), 3-sulfolene and 1,3,5-trimethoxybenzene were purchased from Merck, Stockholm, Sweden. The chemical structures of substances **1**–**15** are depicted in Chart 1.

Disposable 5 mm NMR tubes, HIP-7, as well as deuteriumoxide ($D_2O$, 99.8%), dimethylsulfoxide-$d_6$ (DMSO-$d_6$, 99.9%), methanol-$d_4$ ($CD_3OD$, 99.8%) and chloroform-*d* ($CDCl_3$, 99.8%) with the chemical formulae and degrees of deuteration in parentheses, manufactured by Armar Chemicals, Döttingen, Switzerland, were purchased from Glaser Lab-Kemikalier, Göteborg, Sweden. The paracetamol 500 mg medicinal product was obtained from Apoteket AB, Sweden.

### 2.2. NMR sample preparation

The NMR samples used for determination of spectral properties and $T_1$ relaxation times were prepared by dissolving a few milligrams of the respective internal standard in *ca.* 0.8 ml deuterated solvent, followed by agitation on a vibrating test tube shaker until the solutions were clear. For the compounds dissolving poorly, *ca.* 10-min sonication treatment in ultrasonic bath was used. Finally the clear solutions were transferred into NMR tubes.

Samples for purity determination were prepared by precise weighing of 5–10 mg of the selected substance and *ca.* 5 mg of acetanilide on separate aluminum pans using a Mettler-Toledo UMT2 (Mettler-Toledo GmbH, Greifensee, Switzerland) microbalance with a resolution of 0.0001 mg. Both pans were transferred to a 10 ml glass sample tube, and 0.8 ml deuterated solvent was added. The mixture was shaken, sonicated, and finally the solution was transferred into a disposable NMR tube. Six weighings were performed for each substance.

For the commercially available 500 mg paracetamol product sample, one tablet was weighed and, afterwards, pounded on a mortar. Every sample was made of *ca.* 12 mg powder and 5 mg of the respective internal standard. Each mixture was then dissolved in *ca.* 1.0 ml of DMSO-$d_6$, shaken, sonicated and the solution transferred into an NMR tube. Three weighings were performed for each internal standard.

### 2.3. NMR instrumentation

All $^1H$ NMR spectra were acquired at 25 °C using a Bruker Avance 300 spectrometer operating at 300.13 MHz. The spectrometer was equipped with a 5 mm Z-gradient BB probe using a $^1H$ 90° pulse width of 7.2 μs. Processing and spectra handling was performed using Topspin 1.3 program suite (Bruker Biospin GmbH, Rheinstetten, Germany).

The experimental settings were as follows: excitation frequency 6.175 ppm, spectral width 20 ppm, 64K complex data points, measurement temperature 25 °C, preacquisition delay (de) 6 μs. A 30° pulse was used. Data processing was performed using 64K data

**Table 1**
Spectral properties of the internal standards in different solvents.

| No. | Compound | Mol. Weight (g/mol) | Solvent | | | |
|---|---|---|---|---|---|---|
| | | | $\delta_H$ (ppm), multiplicity[a], number of H[b] | | | |
| | | | $D_2O$ | DMSO-$d_6$ | $CD_3OD$ | $CDCl_3$ |
| **1** | 3,4,5-Trichloropyridine | 182.44 | 8.44 (s) [2][c] | 8.77 (s) [2] | 8.61 (s) [2] | 8.53 (s) [2] |
| **2** | 1,4-Dinitrobenzene | 168.11 | 8.36 (s) [4][c] | 8.46 (s) [4] | 8.47 (s) [4] | 8.44 (s) [4] |
| **3** | 1,3,5-Trichloro-2-nitrobenzene | 226.45 | [d] | 8.12 (s) [2] | 7.80 (s) [2] | 7.47 (s) [2] |
| **4** | Dimethyl terephthalate | 194.19 | 3.85 (s) [6][c] | 3.89 (s) [6] | 3.94 (s) [6] | 3.96 (s) [6] |
| | | | 8.02 (s) [4][c] | 8.09 (s) [4] | 8.11 (s) [4] | 8.11 (s) [4] |
| **5** | Maleic acid | 116.07 | 6.21 (s) [2] | 6.03 (s) [2] | 6.26 (s) [2] | [d] |
| **6** | 2,4,6-Triiodophenol | 471.80 | [d] | 7.97 (s) [2] | 7.97 (s) [2] | 7.94 (s) [2] |
| | | | | 9.72 (s) [1] | | 5.77 (s) [1] |
| **7** | 2,3,5-Triiodobenzoic acid | 499.81 | 7.41 (d) [1][c] | 7.72 (d) [1] | 7.75 (d) [1] | 7.94 (d) [1][c] |
| | | | 8.19 (d) [1][c] | 8.31 (d) [1] | 8.35 (d) [1] | 8.37 (d) [1][c] |
| **8** | Fumaric acid | 116.07 | 6.73 (s) [2] | 6.63 (s) [2] | 6.76 (s) [2] | [d] |
| | | | | 13.12 (s) [2] | | |
| **9** | Acetanilide | 135.16 | 2.05 (s) [3] | 2.03 (s) [3] | 2.12 (s) [3] | 2.19 (s) [3] |
| | | | 7.15 (m) [1] | 7.01 (t) [1] | 7.10 (t) [1] | 7.12 (t) [1] |
| | | | ~7.3 (m) [4] | 7.28 (t) [2] | 7.30 (t) [2] | 7.25 (s) [1] |
| | | | | 7.56 (d) [2] | 7.53 (d) [2] | 7.33 (t) [2] |
| | | | | 9.91 (s) [1] | | 7.51 (d) [2] |
| **10** | Dimethyl sulfone | 94.13 | 3.03 (s) [6] | 2.99 (s) [6] | 3.01 (s) [6] | 2.99 (s) [6] |
| **11** | 3,4,5-Trimethoxybenzaldehyde | 196.20 | 3.76 (s) [3] | 3.76 (s) [3] | 3.86 (s) [3] | 3.95 (m) [9] |
| | | | 3.82 (s) [6] | 3.86 (s) [6] | 3.92 (s) [6] | 7.14 (s) [2] |
| | | | 7.19 (s) [2] | 7.26 (s) [2] | 7.25 (s) [2] | 9.88 (s) [1] |
| | | | 9.67 (s) [1] | 9.88 (s) [1] | 9.85 (s) [1] | |
| **12** | Hexamethylcyclotrisiloxane | 222.46 | 0.06 (s) [18][c] | 0.13 (s) [18] | ~0.1[e] | 0.18 (s) [18] |
| **13** | 2,2-Dimethylmalonic acid | 132.11 | 1.32 (s) [6] | 1.27 (s) [6] | 1.39 (s) [6] | [d] |
| | | | | 12.61 (s) [2] | | |
| **14** | 2,4,5-Trichloropyrimidine | 183.42 | 8.67 (s) [1] | 9.04 (s) [1] | 8.79 (s) [1] | 8.61 (s) [1] |
| **15** | Sodium acetate | 82.03 | 1.80 (s) [3] | 1.58 (s) [3] | 1.89 (s) [3] | [d] |

[a] Splitting pattern of the signal; (s) singlet, (d) doublet, (t) triplet, (m) multiplet or overlap.
[b] Number of equivalent protons in the signal.
[c] Very limited solubility.
[d] Insoluble.
[e] Multiple peaks observed.

points and an exponential line broadening factor of 0.3 Hz. The chemical shifts were reported in parts per million (ppm) and all the spectra were referenced to the residual proton signals of the respective solvent: $\delta_H$ 4.67 for $D_2O$, $\delta_H$ 2.50 for DMSO-$d_6$, $\delta_H$ 3.31 for $CD_3OD$, $\delta_H$ 7.27 for $CDCl_3$.

Relaxation time measurements, using the inversion recovery technique, were performed under automation using 16 different tau delays ranging from 0.001 to 5 s, a repetition delay of 50 s, 8 FID repetitions, 16K complex acquisition data points, 8K processing data points, 1.0 Hz line broadening factor, and non-linear fit of peak intensities.

### 2.4. Quantification by NMR

In order to ensure reliable results for the purity determinations a repetition delay of 90 s was used, and 64 FID repetitions (number of scans) resulted in a suitable signal-to-noise ratio. The other experimental settings were as stated above for $^1$H NMR spectra. The measurement time was ~100 min per sample.

Phasing and integration of the spectrum was performed manually, and the start and end points of each integral region were forced to zero amplitude using a fifth order polynomial baseline correction algorithm. Integration regions around the signals of interest were selected to cover the entire frequency interval between the two carbon satellite signals, and did, in all cases, suffice a substantially wider range than the required 32× the signal half width on both sides [15]. Each integral edge was checked to be almost horizontal and, if needed, manual bias and/or slope correction was applied to the integral.

The area of the signals appearing on a 1D $^1$H spectrum is directly proportional to the number of protons present in the active volume of the sample. Hence, using Eq. (1), all the determinations in this study were performed on 1D $^1$H NMR spectra through the proportional comparison of the peak areas integrated for both the selected signal from the internal standard and from the substance in question:

$$m_{(x)} = P_{(\mathrm{std})} \cdot \frac{MW_{(x)}}{MW_{(\mathrm{std})}} \cdot \frac{nH_{(\mathrm{std})}}{nH_{(x)}} \cdot \frac{m_{(\mathrm{std})}}{P_{(x)}} \cdot \frac{A_{(x)}}{A_{(\mathrm{std})}} \quad (1)$$

where $m_{(x)}$ and $m_{(\mathrm{std})}$ are the masses (weights) in g, $MW_{(x)}$ and $MW_{(\mathrm{std})}$ are the molecular weights in g/mol, $P_{(x)}$ and $P_{(\mathrm{std})}$ are the purities, $nH_{(x)}$ and $nH_{(\mathrm{std})}$ are the number of protons generating the selected signals for integration, $A_{(x)}$ and $A_{(\mathrm{std})}$ are the areas for the selected peaks of the analyte and the internal standard, all respectively.

### 2.5. Differential scanning calorimetry (DSC)

DSC measurements were performed on a Q1000 DSC instrument (TA Instruments, DE, USA) equipped with a refrigerated cooling system and calibrated using an indium metal standard (99.999%). Three samples of each substance (internal standard) were weighed (1.7–2.6 mg) in aluminum pans. Melting curves were recorded using encapsulated sample and empty reference pans, a sampling rate of 2.0 °C/min, a sampling interval of 0.10 s/point, and a start temperature ~10 °C below the melting point of the sample. Purity calculations were performed using the purity analysis function as described in TA Instruments Explorer software, that operates according to the ASTM procedure E0928 [16].

## 3. Results and discussion

### 3.1. The investigated compounds as internal standards

A suitable internal standard should meet the criteria given below: (i) ability of providing unique and stable signals (chemical shifts), (ii) available purity, (iii) solubility in different NMR solvents, (iv) easily weighable, (v) non-volatile, (vi) non-reactive, (vii) long-term stable, and (viii) having optimum molecular weight (small molecular weight compounds require very small amounts).

Facing the difficulty of finding a suitable internal standard for a qNMR application, there exist only limited and fragmented information in the literature. There are numerous individual studies where only single type of applications were reported [17–19], whereas papers including several internal standards are rare [5,6]. Filling this important gap, the chemical shift ($\delta$) values, assignments, multiplicities and number of protons (for each signal), appropriateness of 25 compounds in four different deuterated solvents ($D_2O$, DMSO-$d_6$, $CD_3OD$, $CDCl_3$) were studied. Some of these compounds were previously used in NMR quantification, while others are novel. The criteria for the selection/ranking of these compounds were chosen on the basis of needs/requirements in our laboratory. Major criteria were unique chemical shift, sharp signal (preferably one singlet), solubility properties, easy-to-use (weighing). Minor selection criteria were reactivity towards the analyte/solvent (inertness), protolytic properties, purity, and toxicity. Criteria such as complexation potential, long-term stability, and extraction behavior were, however, not included in the selection/ranking procedure in this study.

The suitability of the individual compounds as internal standards was examined using different solvents. The spectral properties of the 15 highest ranked compounds were summarized (Table 1), and the individual suitability of each of these compounds based on the criteria given above is discussed below.

*3,4,5-Trichloropyridine* (**1**) was readily soluble in DMSO-$d_6$, $CD_3OD$, and $CDCl_3$, but almost insoluble in $D_2O$. It was an easily weighable, crystalline compound that shows $^1H$ spectra with only one singlet originating from two protons in a normally signal-free region at *ca.* 8.6 ppm. The compound is a very weak protolyte, reasonably some orders of magnitude weaker base than pyridine [20], *i.e.*, the $^1H$ NMR chemical shift should not be effected due to protolytic activity in the sample.

*1,4-Dinitrobenzene* (**2**) was soluble in DMSO-$d_6$, $CD_3OD$, $CDCl_3$, but almost insoluble in $D_2O$. The $^1H$ spectrum showed only one sharp singlet, originating from four protons at *ca.* 8.4 ppm. The substance is considered toxic and harmful for the environment.

*1,3,5-Trichloro-2-nitrobenzene* (**3**) was soluble in all solvents except for $D_2O$. The molecule contains only two aromatic protons, which appeared as one sharp singlet at around 8.0 ppm. Notably, the chemical shift varied much between different solvents. The substance is considered toxic.

*Dimethyl terephthalate* (**4**) was easily soluble in DMSO-$d_6$, $CD_3OD$, and $CDCl_3$, but almost insoluble in $D_2O$. The proton spectra showed two sharp signals as singlets, one at *ca.* 8.1 ppm and another at *ca.* 3.9 ppm. The signal of the methyl group at around 3.9 ppm had an unexceptional chemical shift in a normally signal-crowded area, whereas the low-field signal at around 8.1 ppm (4H) was of high potential to be employed as reference signal.

*Maleic acid* (**5**) was soluble in all solvents except $CDCl_3$. Two signals appeared in the proton spectra of maleic acid: one sharp singlet at around 6.1 ppm, and a broad one for the carboxylic acid protons at around 11.0 ppm. Although the compound is hygroscopic, maleic acid was a good choice for the quantification analyses giving the credits to its distinct solubility in $D_2O$ and easy handling, *i.e.*, easily weighable crystalline. Maleic acid is a protolyte, *i.e.*, when mixed with other protolytes, the chemical shift of the signal may change to some extent.

*2,4,6-Triiodophenol* (**6**) was soluble in all solvents except for $D_2O$. It showed a valuable reference signal at around 7.95 ppm, being constituted by two protons. The phenolic proton appeared at about 9.8 ppm as a broad peak. The compound is a weak protolyte.

*2,3,5-Triiodobenzoic acid* (**7**) was easily soluble in DMSO-$d_6$ and $CD_3OD$; and partly soluble in $CDCl_3$ and $D_2O$. The proton spectra showed two doublets and one broad acidic proton signal. One of the doublets showed a unique chemical shift at around 8.35 ppm, suitable for quantification, whereas the broad acidic signal was ignored due to its nature. The compound is a protolyte, *cf.*, maleic acid above.

*Fumaric acid* (**8**) was soluble in DMSO-$d_6$, $CD_3OD$ but not in $CDCl_3$. It required heat and sonication to dissolve in $D_2O$. The proton spectra showed a singlet at around 6.6. Only in the spectrum of fumaric acid in DMSO-$d_6$, it was possible to observe the singlet from the carboxyl protons resonating at 13.12 ppm. Fumaric acid is a protolyte, *cf.*, maleic acid above.

*Acetanilide* (**9**) is a white to grey solid that was soluble in DMSO-$d_6$, $CD_3OD$ and $CDCl_3$, and sparingly soluble in $D_2O$. It exhibited four signals and three of them were in the region where typically aromatic proton signals are found. Acetanilide was of high purity analytical grade and reasonably prized.

*Dimethyl sulfone* (**10**) was soluble in all employed solvents. However, the substance was not suitable as internal standard due to low molecular weight and the fact that the signal in the spectrum comprised six protons. Probably the error in quantification will be higher compared to other suitable internal standards. A signal at 3.0 ppm was not unique and also prone to prospective overlap problems.

*3,4,5-Trimethoxybenzaldehyde* (**11**) was soluble in DMSO-$d_6$, $CD_3OD$ and $CDCl_3$, and partly soluble in $D_2O$. At first glance the spectra showed a unique aldehyde signal of one proton at *ca.* 9.8 ppm, which seemed to be advantageous. However, being an aldehyde, this compound awakened some concerns about stability and reactivity properties, requiring further investigations.

*Hexamethylcyclotrisiloxane* (**12**) was soluble in all solvents apart from $D_2O$. This moisture sensitive and unstable compound had one signal at *ca.* 0.1 ppm, sporting a unique chemical shift outside of the most crowded regions. However, it contained 18 protons exerting a source of weight error for quantification.

*2,2-Dimethylmalonic acid* (**13**) was soluble in all solvents apart from $CDCl_3$. Two signals appear in the spectrum: 1.3 and 12.6 ppm. The methyl signal at 1.3 ppm was not unique and the signal of acidic protons at 12.6 ppm was too broad. The compound is a protolyte, *cf.*, maleic acid above.

*2,4,5-Trichloropyrimidine* (**14**) was soluble in DMSO-$d_6$, $CD_3OD$ and $CDCl_3$, and partly soluble in $D_2O$. It showed a suitable singlet signal at around 9.0 ppm. The only drawback was that this compound is a liquid, requiring careful handling during weighing. The compound is a protolyte, *cf.*, 3,4,5-trichloropyridine above.

*Sodium acetate* (**15**) is known to be hygroscopic. It was soluble in $CD_3OD$ and $D_2O$, partly soluble in DMSO-$d_6$ but insoluble in $CDCl_3$. Furthermore, it is well known that NMR tubes as well as other chemical lab ware may contain acetate ions as impurities, which means that a full investigation of blank samples need to be performed. The chemical shift of the signal was around 1.8 ppm, which was not unique as to avoiding overlap. The compound is a protolyte, *cf.*, maleic acid above.

The above 15 compounds were found to meet many of the qualities of a useful internal standard. We also studied other compounds (hexamine, uracil, L-leucine, anthracene, acetamide, 2,3,4-trifluorobenzamide, 2,4,6-trifluorobenzoic acid, 2,4,6-trichlorobenzoyl chloride, 2,3,5-tribromothiophene, 5-fluorouracil), that showed out not to be particularly suitable as internal standards due to various reasons, *e.g.*, non-unique

**Table 2**
Purities of the selected internal standards by NMR, DSC, and as reported by the certificate of analysis.

| Compound | NMR impurity peaks | NMR[a] purity, std. dev. | DSC[b] purity, std. dev. | Certificate of analysis, technique |
|---|---|---|---|---|
| **1** | –[c] | 99.5 ± 0.3% | 99.9 ± 0.06% | 99.9%, GC |
| **2** | –[c] | 98.1 ± 0.6% | 99.6 ± 0.03% | 99.9%, GC |
| **3** | $\delta_H$ 8.6, area ~1% | 97.8 ± 0.1% | 98.0 ± 0.05% | 98.7%, GC |
| **4** | –[c] | 100.0 ± 0.3% | 99.9 ± 0.02% | 100.0%, GC |
| **5** | –[c] | 99.6 ± 0.3% | –[d] | 100.0%, HPLC |
| **6** | $\delta_H$ 1.1, area ~1% | 96.5 ± 0.5% | 98.8 ± 0.14% | 97.8%, GC |
| **7** | $\delta_H$ 7.6, area ~2% | 99.8 ± 1.2% | 99.5 ± 0.03% | 99.3%, HPLC |
| **8** | –[c] | 99.2 ± 0.2% | –[d] | 100.0%, HPLC |
| **9** | –[c] | –[d] | 99.9 ± 0.03% | 100.0%[e] |

[a] Weight%, $n = 6$ ($n = 5$ for compound 3).
[b] mol%, $n = 3$.
[c] No obvious impurity peaks larger than ~0.1% detected.
[d] Not determined.
[e] Analytical technique not reported in the certificate.

chemical shift(s), broad/heavily "splitted" signals, toxicity, and reactivity/non-stability.

Based on these observations on the aptness of the individual compounds as internal standards, compounds **1**–**8** were studied further in order to determine their absolute purity using both differential scanning calorimetry (DSC) and quantitative NMR spectroscopy. The highly pure acetanilide was employed as the reference internal standard.

### 3.2. Acetanilide as control reference for qNMR

In order to perform accurate quantification it is essential to know the actual purity of the used internal standard. Suitable methods for determination of purity are DSC and qNMR. The performance of qNMR has been thoroughly assessed in previous studies [10,21,22]. Using qNMR it is necessary to use an established reference standard of high purity, *e.g.*, acetanilide. The reference standard itself can advantageously be qualified by $^1$H NMR spectroscopy, with respect to observed organic impurities, in combination with measurement of the water content by, *e.g.*, loss-on-drying, and inorganic impurities by, *e.g.*, sulphated ash.

In the current work, acetanilide due to its high level of purity (reported as 100.0%) and renowned stability was selected as the reference standard to be used in purity determination of the eight internal standards chosen above [23]. Quantification was performed on mixtures of acetanilide and the respective analyte in DMSO-$d_6$, using integrals of the acetanilide signal at 2.0 ppm and the analyte signal of highest chemical shift.

In order to exemplify the employment of acetanilide in purity determination, two frequently used internal standards, 3-sulfolene and 1,3,5-trimethoxybenzene, were checked for their long-term purity stabilities using the qNMR protocol described in Section 2. The compounds were more than 3 years old and kept in dry environment in their original containers with tightly closed caps. The observed purity was 98.9% for 3-sulfolene, RSD 0.2% ($n = 6$), and 99.2% for 1,3,5-trimethoxybenzene, RSD 0.2% ($n = 6$). No obvious impurity peaks, except for water, were detected in the $^1$H NMR spectra. The measured differential scanning calorimetry (DSC) assay at time of delivery was 99.9% for both compounds. The fact that lower assays were obtained after storage of these compounds show on the necessity to implement a quick assay check method for internal standards used in routine work.

### 3.3. Purities of the selected internal standards

The purities of the eight selected compounds and acetanilide were first determined using DSC. The analyses performed smoothly for compounds **1**, **2**, **3**, **4**, **6**, and **7** as well as for acetanilide (**9**). Unfortunately, the analysis failed for maleic acid (**5**) and fumaric acid (**8**). The reason might be conversion of maleic to fumaric acid near the melting point, sublimation and/or the formation of the anhydride during heating. The results from DSC analyses (Table 2) were reported as mol%. The relative standard deviation (RSD) was excellent indicating that the content was homogeneous for all compounds.

Purity determination by qNMR was performed for each of the eight selected internal standards using the acetanilide method described above (Table 2).

In most cases similar results were obtained using NMR and DSC, and the results also correlated well with the purities reported in the certificates. The fact that qNMR purities, unlike the other techniques, are reported as weight percentages, influence the reported values. High molecular weight impurities would result in lower qNMR purities as compared to, *e.g.*, DSC mol percentage purities. For

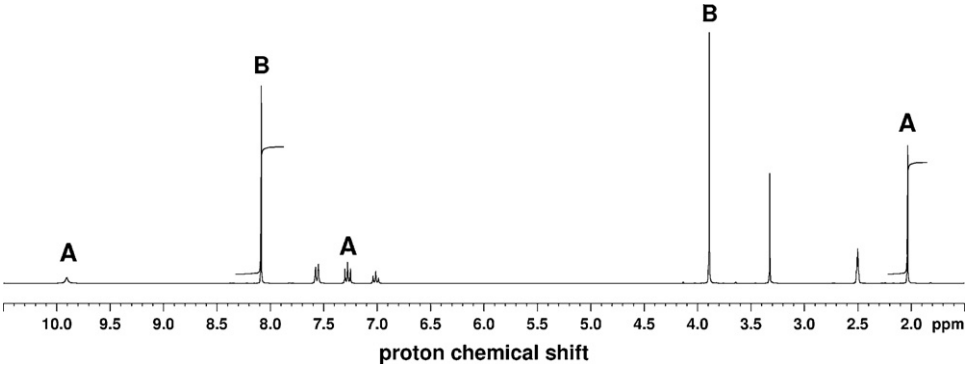


**Fig. 1.** $^1$H NMR spectrum from purity determination of dimethyl terephthalate (B) using 100% pure acetanilide (A) as a primary reference standard. The areas of the signals at 8.1 ppm (B) and 2.0 ppm (A) were used for purity calculation. Residual solvent peaks from DMSO-$d_6$ and water appear at 2.5 and 3.3 ppm, respectively.

low molecular weight impurities, *e.g.*, residual solvents, the qNMR purity would be higher than the corresponding mol percentage purity. In previous comparative purity studies, determination by the traditional mass-balance HPLC method and the $^1$H NMR method showed similar absolute purities [24,25] for compounds with 90% or higher purities.

The results (Table 2) showed that compounds **1**, **4** and **5** were highly pure and the qNMR assays resulted in purities of 99.5%, 100.0%, and 99.6%, respectively, similar to the results obtained by DSC purities and the reported value in the product certificates. Only few tiny impurity peaks, much smaller than the $^{13}$C-satellites, were present in the proton spectra. An illustrative spectrum for dimethyl terephtalate is shown in Fig. 1.

Compounds **2** and **8** showed no obvious impurity peaks and the obtained qNMR purities were 98.1% and 99.2%, respectively, which were lower compared to purities obtained from DSC and the certificate. The reason may be due to difference in the water content, but this was not further studied.

For compounds **3** and **7** similar results were obtained using the different techniques, but for compound **6** the result of the qNMR assay was lower compared to the results from the DSC and certificate assays. Obvious impurity peaks of 1–2 area% were observed in the proton spectra of **3**, **6** and **7** (Table 2). Compared to the other compounds, compound **7** showed a low precision in the qNMR purity determination. This may be due to problems with hygroscopicity, poor solution stability, poor substance homogeneity, or other undetermined issues in sample preparation or NMR analysis, and must be considered.

### 3.4. Spin-lattice relaxation times ($T_1$)

The proton spin-lattice relaxation times ($T_1$) of the eight selected internal standards were measured on the 300 MHz spectrometer using a standard inversion recovery experiment [26]. The resulting exponential curves have been analyzed, and the $T_1$ values of the protons of the selected compounds are reported in Table 3.

Employing an insufficiently short delay between the repeated NMR experiments (scans) in qNMR would cause incorrect quantification results. Usually it is accustomed to use a minimum delay time of five times the longest corresponding $T_1$ value [15]. The longest $T_1$ 11.3 s was obtained for 3,4,5-trichloropyridine (**1**) in DMSO-$d_6$. This means that a repetition delay of 57 s is required in order to ensure reasonable relaxation between 90° pulses. For dimethyl terephthalate (**4**), on the other hand, at a maximum $T_1$ 4.1 s was observed in $CD_3OD$ for the aromatic proton and, accordingly, a repetition delay of 21 s would be sufficient. Consequently, a quickly relaxing standard would result in a shorter measurement time, assuming quick relaxation of the analyte protons. It should be noted that for a smaller pulse angle, e.g., 30°, a shorter repetition delay can be used in order to maximize sensitivity in a fixed amount of instrument time [15]. Since the routine qNMR method in our laboratory involves a 30° pulse angle and 90 s repetition delay, the complete relaxation criterion between scans was fulfilled for all of these eight standards.

**Table 3**
$^1$H relaxation times ($T_1$) in seconds of the selected internal standards in various NMR solvents.

| Compound[a] | $D_2O$ (s) | DMSO-$d_6$ (s) | $CD_3OD$ (s) | $CDCl_3$ (s) |
|---|---|---|---|---|
| **1** | –[b] | 11.3 | 6.8 | 6.6 |
| **2** | –[b] | 5.2 | 6.2 | 5.8 |
| **3** | –[b] | 10.2 | 7.3 | 7.2 |
| **4** | –[b] | 1.1[c], 2.7[d] | 2.4[c], 4.1[d] | 2.0[c], 3.8[d] |
| **5** | 6.7 | 2.4 | 4.0 | –[b] |
| **6** | –[b] | 4.4 | 6.1 | 6.8 |
| **7** | –[b] | 4.5[e], 4.5[f] | 3.7[e], 4.4[f] | –[b] |
| **8** | 10.0 | 4.4 | 5.2 | –[b] |

[a] Concentration 2–5 mg/ml.
[b] Not determined due to low solubility.
[c] $\delta_H$ 3.9–4.0.
[d] $\delta_H$ 8.1.
[e] $\delta_H$ 7.7–7.9.
[f] $\delta_H$ 8.3–8.4.

### 3.5. Employment of the selected internal standards in a real case test

Selected internal standards were used for the quantification of paracetamol (acetaminophen, *N*-(4-hydroxyphenyl)acetamide) in a 500 mg paracetamol tablet. All samples were portions taken from pulverized material from one and single tablet, DMSO-$d_6$ was used in order to ensure maximum recovery of paracetamol and the internal standard, and quantification was made by integration of the methyl signal of paracetamol at *ca.* 2.0 ppm and a non-overlapping signal of the respective internal standard. A typical example spectrum is shown in Fig. 2.

Different purities of the standards obtained by NMR and DSC and from the certificate of analysis, reported in Table 2 above, were used for the assay calculations. The results are reported in Table 4.

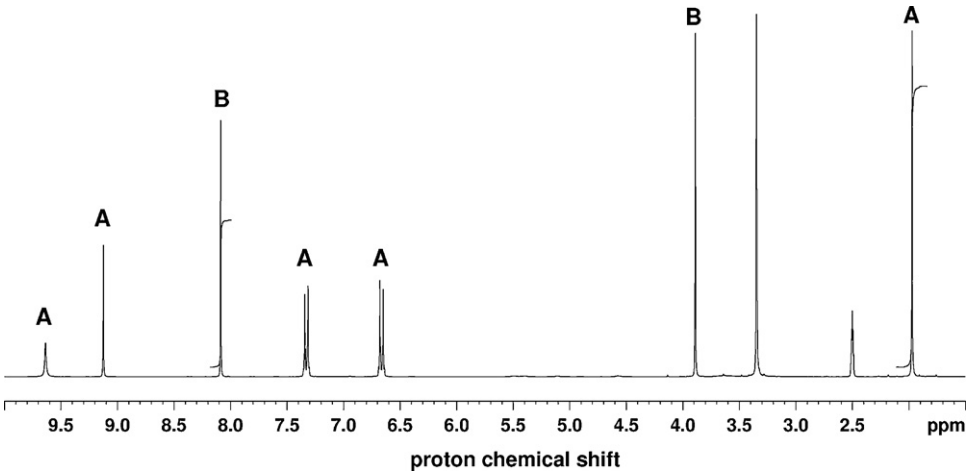


**Fig. 2.** Representative $^1$H NMR spectrum from the determination of paracetamol (A) content in a paracetamol 500 mg tablet, using dimethyl terephthalate (B) as internal standard. The areas of the signals at 8.1 ppm (B) and 2.0 ppm (A) were used for content calculation. Residual solvent peaks from DMSO-$d_6$ and water appear at 2.5 and 3.3 ppm, respectively.

**Table 4**
Determination of paracetamol content in a 500 mg paracetamol tablet in DMSO-$d_6$ using different internal standards, for which purity was determined by different methods as reported in Table 2.

| Internal standard used | Paracetamol content (mg) | | | |
|---|---|---|---|---|
| | NMR[a] | DSC[b] | Certificate[c] | RSD[d] |
| **1** | 517 | 519 | 519 | 1.4% |
| **2** | 518 | 526 | 528 | 1.0% |
| **3** | 527 | 528 | 532 | 2.9% |
| **4** | 517 | 517 | 517 | 0.7% |
| **5** | 514 | –[e] | 516 | 0.4% |
| **6** | 515 | 527 | 522 | 0.9% |
| **7** | 519 | 518 | 517 | 1.3% |

[a] Content calculated using the NMR purity of the standard.
[b] Content calculated using the DSC purity of the standard.
[c] Content calculated using the certificate purity of the standard.
[d] Three weighings were performed for each determination.
[e] Not determined.

The assay of paracetamol fell well within the limits for a single tablet determination [27], and varied from 514 mg/tablet for maleic acid using the NMR purity of the standard, to 532 mg/tablet for 1,3,5-trichloro-2-nitrobenzene using the certificate purity. The latter compound showed the largest measurement uncertainty among the studied standards (RSD 2.9%, $n = 3$).

The fact that different standards produce different results may be due to uncertain purity, which in turn emphasizes the need for proper qualification of the standard prior to utilization. The lowest, as well as most homogenous paracetamol assays were obtained when the purity of the respective standard was certified by NMR spectroscopy (514–527 mg/tablet; Table 4). Somewhat higher content of paracetamol and lower precision were observed using DSC purities (517–528 mg/tablet) and certificate purities (516–532 mg/tablet). In summary, a slight better homogeneity and, in general, a somewhat lower paracetamol content was obtained when the NMR purity was used for calculation. An NMR spectrum would also add information, such as chemical shifts and magnitudes, about possible impurity peaks that may interfere within the integration region of the analyte. If no NMR purity is available, it is recommended that the qNMR internal standard to be used is more than 99% pure, in order to achieve maximum accuracy.

## 4. Conclusion

In this work, a series of 25 ordinary organic compounds were studied for their aptness as internal standards. Out of these, eight internal standards (2,4,6-triiodophenol, 1,3,5-trichloro-2-nitrobenzene, 3,4,5-trichloropyridine, dimethyl terephthalate, 1,4-dinitrobenzene, 2,3,5-triiodobenzoic acid, maleic acid and fumaric acid) were tested and considered to be suitable for routine qNMR applications. None of these internal standards is an ideal or universal standard, but the availability of this set of standards would enable the quantification of almost all prospective drug (or similar) samples requested, since these eight compounds represent a broad diversity both in providing a comprehensive range of different chemical shifts and in supplying the necessary physical properties including solubility in four different common NMR solvents, *i.e.*, $D_2O$, DMSO-$d_6$, $CD_3OD$, $CDCl_3$.

Purity determination of the standards was easily performed using highly pure acetanilide as a qNMR control reference. Moreover, characterization by NMR and DSC in comparison to the certificated purities, and application in quantification of a paracetamol product, yielded insight into the appropriateness of the standards for applicability in qNMR. It was evident that careful purity/impurity control of the standard by NMR is to be recommended instead of characterization by other techniques of analysis.

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