Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 813–823
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Validation of quantitative NMR

F. Malz *, H. Jancke

Federal Institute for Materials Research and Testing, Richard-Willstätter-Str. 11, 12489 Berlin, Germany

Received 24 September 2004; received in revised form 20 January 2005; accepted 20 January 2005
Available online 11 May 2005

Abstract

NMR is by definition a quantitative spectroscopic tool because the intensity of a resonance line is directly proportional to the number of resonant nuclei (spins). This fact enables, in principle, a precise determination of the amount of molecular structures and, hence, of substances in solids as well as liquids. With the increase of sensitivity due to stronger and stronger static magnetic fields including improved electronics the detection limits have been pushed down significantly. However, the lack of a precise protocol that considers and controls the aspects of both the measurement procedure as well as the spectra processing and evaluation is responsible for the fact that quantitative investigations of identical samples in various laboratories may differ severely (deviations up to 90% relative to gravimetric reference values).

Here, a validated protocol for quantitative high resolution ^{1}H-NMR using single pulse excitation is described that has been confirmed by national and international round robin tests. It considers all issues regarding linearity, robustness, specificity, selectivity and accuracy as well as influences of instrument specific parameters and the data processing and evaluation routines. This procedure was tested by the investigation of three different 5-model-compound mixtures. As a result of the round robin tests using the proposed protocol it was found that the maximum combined measurement uncertainty is 1.5% for a confidence interval of 95%. This applies both for the determination of molar ratios and of the amount fractions of the various components. Further, the validation was extended to purity determinations of substances as shown for 1,8-epoxy-*p*-menthane (cineole).
© 2005 Elsevier B.V. All rights reserved.

Keywords: Validation; Quantitative NMR; Cineole; Purity; Round robin test

1. Introduction

Nuclear magnetic resonance spectroscopy (NMR) is one of the most important and widespread analytical methods in academic and industrial research. It enables a unique and, in principle, quantitative determination of the relative amount of molecular groups, thus offering a tool to quantify entire molecular structures even in mixtures. The first quantitative measurements (qNMR) have been described in the literature in 1963 by Jungnickel and Forbes [1] and Hollis [2]. In the first case the intramolecular proton ratios in 26 pure organic substances have been determined whereas Hollis have analyzed the amount fractions of three analytes aspirin, phenacetine and caffeine in respective mixtures. Meanwhile, qNMR has found widespread applications although the disadvantage of high costs for NMR device, particularly due to specific advantages like, (i) the possibility to determine structures at a molecular level, (ii) no need for intensity calibrations in case of determination of ratios (signal area is directly proportional to the number of nuclei), (iii) relatively short measuring times, (iv) its non-destructive character, (v) no prior isolation of the analyte in a mixture, which means rather easy sample preparation and handling and (vi) the possibility of a simultaneous determination of more than one analyte in a mixture. Despite limited accuracy so far, quantitative ^{1}H and ^{13}C NMR in liquids is used e.g. in pharmacy [3–9], in agriculture [10–13], in material science [14], for military purposes [15] etc., where purity or content determinations of substances are the key issues. This development has been accelerated by the substantial increase of the sensitivity and homogeneity of high-field NMR spectrometers as well as by modern software packages that allow an accurate and precise data processing and evaluation.

* Corresponding author.
E-mail address: frank.malz@bam.de (F. Malz).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.01.043

In 1998, Jancke [16] and later the committee for chemical measurements (Comité Consultatif pour la Quantité de Matière, CCQM) [17,18] have discussed the potential of qNMR as a primary method according to the definition of the CCQM [19] based on the fact that the NMR signal response (more precisely the integrated signal area) is directly proportional to the number of nuclei contributing to the signal.

Simultaneously, Maniara et al. [20] and Wells et al. [10,11] published an uncertainty of the result of 0.5% for quantitative high-resolution ^{1}H and ^{31}P NMR measurements. This value is comparable with HPLC data as the standard analytical tool. However, their result included only the own experiments, i.e. of one laboratory. Hence, this uncertainty value is not representative; or in other words, a validation of a method requires the comparison of results of various laboratories. For that reason, first national (NMR-1, [21]) and international (CCQM-4) intercomparisons [22] have been organized by the Federal Institute of Materials Research and Testing (BAM). Including over 30 participants from laboratories of universities, research institutes, companies and national metrology institutes, it was found that the results did differ enormously (up to 100%) not only between the participating laboratories but also with respect to the gravimetric reference values by analysing a simple five model compound mixture. This unacceptable result is mainly caused by the individual and independent setup of the measurements and the data processing and evaluation procedures in each laboratory.

The objective of our efforts was to overcome this serious analytical problem by identifying the various sources of errors in detail. As a result, a protocol for experimental setup of the measurement and the subsequent data processing and handling has been developed and tested intensively using different spectrometer configurations. Additionally, a new national intercomparison (NMR-2) with a large number of participants has been performed and proved the applicability of qNMR as validated method. Also, the validation has been extended to purity determination of selected pharmaceutical substances. This paper focuses on the detailed description of all possible sources of errors, their influence on the final uncertainty budget and, most importantly, on the explanation of the new measurement protocol for qNMR and its first results.

2. Basics

2.1. Quantitative NMR (qNMR)

The basics of high resolution NMR can be found in many textbooks (e.g. [23–27]) and with particular focus on quantitative measurements in [28–30]. The most important fundamental relation of qNMR is that the signal response (integrated signal area) I_x in a spectrum is directly proportional to the number nuclei N_x generating the corresponding resonance line:

$$I_x = K_s N_x \qquad (1)$$

with K_s as a spectrometer constant. Usually the NMR signal of a single substance consists of several resonance lines. However, it is sufficient to select a single resonance line specific for this sample composition. In this case N_x represents the relative number of spins (i.e. protons) which cause this resonance.

The determination of relative area ratios I_x/I_y is the easiest way to obtain quantitative results. For ^{1}H single pulse NMR experiments with correct acquisition parameters (to be discussed later) K_s is the same constant for all resonance lines within the same spectrum, such that it cancels for the ratio:

$$\frac{I_x}{I_y} = \frac{N_x}{N_y}. \qquad (2)$$

The molar ratio n_x/n_y of two compounds X and Y can be calculated straightforward using:

$$\frac{n_x}{n_y} = \frac{I_x}{I_y}\frac{N_y}{N_x}. \qquad (3)$$

Consequently, the amount fraction of a compound X in a mixture of m components is given by:

$$\frac{n_x}{\sum_{i=1}^{m} n_i} = \frac{I_x/N_x}{\sum_{i=1}^{m} I_i/N_i} 100\% \qquad (4)$$

without any need to consider the solvent signal in which the mixture is dissolved as the only sample preparation step.

For the purity determination of a substance an internal standard with known purity is needed. The requirements for such internal standards for qNMR have been described elsewhere [10,31,32]. The purity of the analyte P_x can be calculated as follows

$$P_x = \frac{I_x}{I_{\mathrm{Std}}}\frac{N_{\mathrm{Std}}}{N_x}\frac{M_x}{M_{\mathrm{Std}}}\frac{m_{\mathrm{Std}}}{m} P_{\mathrm{Std}} \qquad (5)$$

where M_x and M_{Std} are the molar masses of the analyte and the standard, respectively, m the weighed mass of the investigated sample, m_{Std} and P_{Std} are the weighed mass and the purity of the standard and N_{Std} and I_{Std} correspond to the number of spins and the integrated signal area of a (typical) NMR line of the standard, as described above.

2.2. Validation

Often the terms validation, certification, characterisation, and verification are used in the same context causing confusion because of improper use. In the seventies, the term validation appeared for the first time (1975 in Europe, 1978 in USA) in the literature. Several different definitions of validation have been described [33]. The combination of the ISO Guide 25 [34] and the EN 45001 [35] to the new international norm DIN EN ISO/IEC 17025 [36] yields a unique definition for the validation of analytical methods as the ‘confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled’ [36]. As a straightforward conclusion non-standard methods, like NMR must be validated. The validation process requires the testing of linearity, robustness, parameters of

accuracy (repeatability, comparability and measurement uncertainty), specificity, and selectivity [37]. Intercomparisons or round robin tests complete the validation.

2.3. Measurement uncertainty

The two international guidelines (GUM [38] and EURACHEM [39]) describe the procedure how the measurement uncertainty for analytical methods has to be determined. According to these two guidelines all uncertainties $u(x_i)$, corresponding to the parameters of the measurement equation must be taken into account (uncertainty propagation). For Eq. (3) the combined uncertainty for the determination of molar ratios $u_c(n_x/n_y)$ is given by:

$$u_c\left(\frac{n_x}{n_y}\right) = \frac{n_x}{n_y}\sqrt{\left(\frac{u(I_x/I_y)}{I_x/I_y}\right)^2} \tag{6}$$

with

$$u\left(\frac{I_x}{I_{Std}}\right) = \sqrt{\frac{\sum_{K=1}^{n}(x_k-\bar{x})^2}{n(n-1)}}, \tag{7}$$

where x_k represents the result of a single measurement k (out of n measurements) whereas $\bar{x}$ is the mean value of these n measurements. This combined uncertainty contains and describes all uncertainties and errors of the whole measurement procedure.

In the case of purity determinations (Eq. (5)) the combined uncertainty $u_c(P_x)$ contains additional quantities and it can calculated by:

$$u_c(P_x) = P_x\sqrt{\left(\frac{u(I_x/I_{Std})}{I_x/I_{Std}}\right)^2 + \left(\frac{u(M_x)}{M_x}\right)^2 + \left(\frac{u(M_{Std})}{M_{Std}}\right)^2 + \left(\frac{u(m_x)}{m_x}\right)^2 + \left(\frac{u(m_{Std})}{m_{Std}}\right)^2 + \left(\frac{u(P_{Std})}{P_{Std}}\right)^2} \tag{8}$$

For the uncertainties of molar masses $u(M)$ the number of atoms N_j of the element j (e.g. C, H, etc.) and the uncertainties of the atom masses $u(j)$ have to be considered as well:

$$u(M) = \sqrt{\sum_{J=1}^{n}(N_j u(j))^2}. \tag{9}$$

Further, the uncertainties of the initial weighings $u(m_i)$ are given by the uncertainty parameters of the used balance in terms of repeatability $u_w(m)$ and non-linearity $u_{\text{non-linear}}(m)$ (these parameters are provided by the manufacturer)

$$u(m_i) = \sqrt{u_w^2(m) + 2u_{\text{non-linear}}^2(m)}. \tag{10}$$

The uncertainty of the purity of the standard $u(P_{Std})$ has to be considered accordingly.

For a true statement on systematic errors Δ (accuracy of the method), the mean values $\bar{x}$ must be checked against reference values x_{Ref} as follows:

$$\Delta_x = \bar{x} - x_{Ref} \tag{11}$$

As next step it must be checked that the following relations holds:

$$|\Delta_x| \le 2u(\Delta_x) = 2\sqrt{u_c(\bar{x})^2 + u(x_{Ref})^2}. \tag{12}$$

If true, the systematic error and the uncertainty of the reference value must be included in the measurement uncertainty $u_c(y)$ for the result y as follows [40]:

$$u_c(y) = \sqrt{u_c(\bar{x})^2 + \Delta_x^2 + u(x_{Ref})^2}. \tag{13}$$

The extended measurement uncertainty U is always connected with a confidence interval:

$$U = ku_c(y). \tag{14}$$

A factor $k = 2$ corresponds to a confidence interval of 95% and will be used in this paper.

If Eq. (12) is not fulfilled (systematic error too large), the whole procedure including the experiment must be reconsidered and improved.

3. Experimental

3.1. Materials

Cyclododecane (Cy_{12}, >99%, Merck), [2,2]-paracyclophane (Par, >99%, Merck), ethyl-4-toluene sulfonate (ETS, >99%, Merck), 1,2,4,5-tetramethyl benzene (Dur, >99%, Merck), octamethylcyclotetrasiloxane (D_4, >98%, Merck), and 1,3-dimethoxy benzene (DMB, >99%, Merck) were used for the validation of the method. The 1,8-epoxy-*p*-menthane (Cineole, HWI) was taken for purity measurements. As solvents deuterated chloroform ($CDCl_3$, >99.8%, Merck), deuterated benzene-d_6 (>99.5%, Merck), and deuterated dimethyl sulfoxide-d_6 (DMSO-d_6, >99,96%, Merck) were used.

3.2. Internal standards

Benzoic acid SRM 350a (certified purity of (99.9958 ± 0.0027) g/g%) NIST, Dimethyl terephthalate (>99%, Merck).

3.3. Model mixtures

For the validation and intercomparisons three model mixtures (one main, four minor compounds) were prepared gravimetrically. For the round robin test NMR-1 the following chemicals have been dissolved in $CDCl_3$ (also assigned as mixture of sample NMR-1): Dur (amount fraction: 81.50 mol/mol%), ETS (13.25 mol/mol%), Cy_{12}

(2.70 mol/mol%), D_4 (2.23 mol/mol%) and DMB (0.351 mol/mol%). Sample CCQM-P3 contains Dur (94.22 mol/mol%), ETS (1.839 mol/mol%), Par (1.253 mol/mol%), Cy_{12} (1.087 mol/mol%) and D_4 (1.601 mol/mol%) dissolved in benzene-d_6. Sample NMR-2 consists of Dur (97.10 mol/mol%), ETS (1.024 mol/mol%mol%), Par (0.702 mol/mol%), Cy_{12} (0.793 mol/mol%) and D_4 (0.380 mol/mol%) dissolved in benzene-d_6.

3.4. Experimental procedure

Most of the measurements were carried out with a Bruker DMX 400 spectrometer with a 5 mm qnp probe at 400.13 MHz (^{1}H). For comparison, a Bruker DPX 300 (5 mm qnp, 300.13 MHz), and a Bruker AMX 600 (5 mm bbi, 600.13 MHz) at the Humboldt-University, Berlin, as well as a Bruker Avance 500 (5 mm txi, 5 mm cryo-txi, 500.12 MHz), a Bruker Avance 600 (5 mm txi, 600.13 MHz) and a Bruker Avance 800 (5 mm txi, 800.25 MHz) at Bruker BioSpin, Rheinstetten were used. In general, the experiments were measured with the following parameters optimised for qNMR: 30° pulse, preacquisition delay of 5 μs, 32 k data points (corresponding to a acquisition time of 3.4 s at a sweep width of 4807 Hz), relaxation delay of 10 s (round robin test NMR-1), 20 s for NMR-2, and 30 s when using pharmaceutical standards and a total of 32 scans. Fourier transformation was done after zero filling the data to 64 k time domain points [40,41] and exponential filtering (em command) of 0,3 Hz. Phase and baseline corrections were done manually. This manual mode was used also for the signal integration (choice of integration limits (generally without the ^{13}C satellites) and if needed the BIAS- and SLOPE-functions for the integral calculation due to improper baseline corrections). For statistical reasons, each measurement was repeated four times.

4. Results of validation and discussion

The validation was performed using simple model compounds having sufficiently separated signals (mostly singulets) in the aliphatic region. Solvents and analytes with purities >99% were used in general. The 90° pulse length and the T_1 relaxation times were determined before. The longest T_1 relaxation time of 4.3 s was determined for ETS. Table 1 summarizes the experimental parameters that allow precise and accurate measurements. This parameter set has been obtained and secured for sample NMR-1 by comparison with the gravimetric reference values.

Table 2
Gravimetric and experimental values of the 13 solutions for linearity testing (molar ratio of D_4 refers to Dur)

Sample no.	Gravimetric value (mol mol^{-1})	Experimental value (mol mol^{-1})
1	98.95	98.96
2	91.08	91.18
3	85.71	85.95
4	73.20	74.03
5	63.07	63.65
6	57.56	58.12
7	50.11	50.28
8	44.34	44.89
9	38.09	38.33
10	29.34	29.56
11	16.57	16.43
12	9.13	9.09
13	2.32	2.29

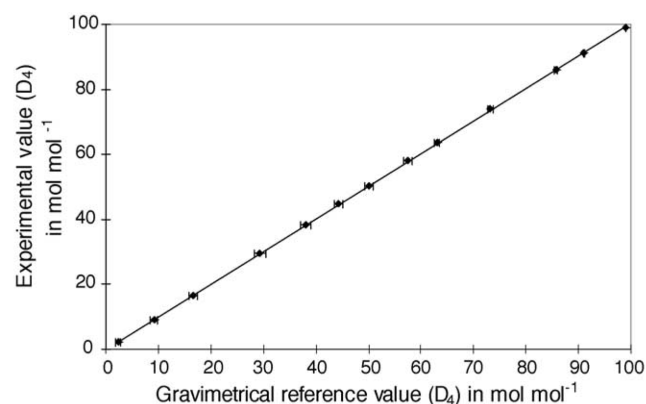

Fig. 1. Test of linearity. Theoretical and experimental molar ratios of the 13 model mixtures, calculated for D_4. Correlation coefficient $r^2 = 0.99992$.

4.1. Linearity

For checking the linearity of the method, 13 model solutions were prepared which contain D_4 and Dur in different molar ratios (from 2.32 to 98.95 mol mol^{-1} for D_4) solved in $CDCl_3$, shown in Table 2.

Fig. 1 shows the experimentally determined molar ratios for D_4 versus the gravimetric reference values.

Table 1
Standard parameters for measurements with model mixture NMR-1

90° pulse strength	2 dB	Number of scans	32
90° pulse length	10 μs	Sweep width	12 ppm (4807 Hz)
Spin rotation	12 Hz	Filter width	90.000 Hz
Measurement temperature	300 K	Filter	Digital
Pulse angle	30°	Number of FID-points	32 k
Preacquisition delay	5 μs	Number of Frequency-points	64 k
Acquisition time	3.4 s	Line broadening	0.3 Hz
Relaxation delay	10 s		

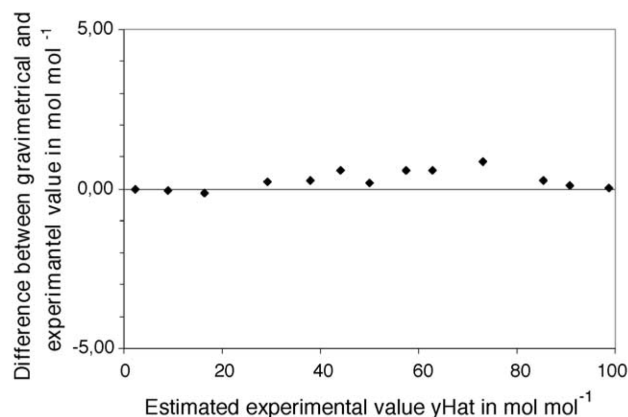

Fig. 2. Test of linearity. Difference between gravimetric and experimental value vs. estimated experimental value yHat (with yHat = 1.004*x*, whereas *x* represents the gravimetric value).

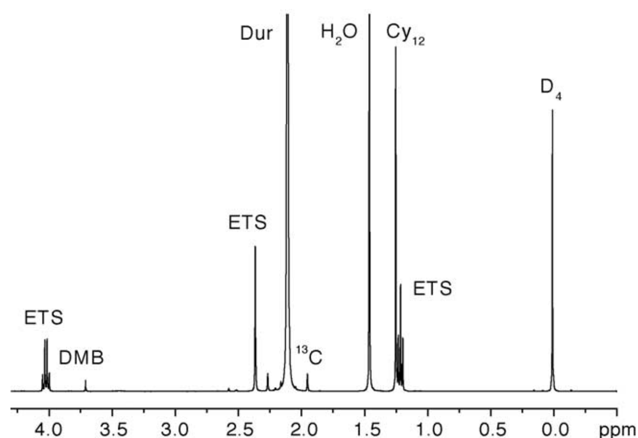

Fig. 3. ^{1}H-NMR of the model system NMR-1. Aliphatic proton signals.

Linear regression yielded a correlation coefficient of 0.99992 and a regression line of $y = 1.004x$. Fig. 2 shows the difference between gravimetric and experimental value versus estimated experimental value yHat (with yHat = 1.004x, whereas x represents the gravimetric value). For a required measurement uncertainty of qNMR of 1% the linearity is confirmed unambiguously, because for each sample the data points differs generally by less than 1% from the corresponding gravimetric value (cf. Table 2).

4.2. Robustness

For the second part of the validation procedure, testing of robustness, three gravimetrically prepared model systems NMR-1, NMR-2 and CCQM-P3 were used. The spectrum in Fig. 3 shows the resonance line of the aliphatic protons of sample NMR-1.

Each of the aliphatic signals can be evaluated separately. If, however, the B_0 field is not shimmed properly two resonance lines of sample NMR-1 (1.33 ppm (Cy_{12}) and 1.29 ppm (ETS)) overlap partially. In this case only the total integral over both lines can be obtained. Therefore, another signal of ETS must be evaluated in order to separate the contributions of the two compounds from that integral value because Cy_{12} has only a single resonance.

For the evaluation of the robustness of the method all important parameters of the data acquisition, processing, and evaluation of the NMR spectra were varied stepwise in wide ranges, starting from the standard sets (Table 1). In order to study the influence of the phase correction on the quantitative result, precisely corrected (phase and baseline) spectra were compared with such spectra, in which phasing errors were implemented intentionally. For the latter case, BIAS and SLOPE were used in order to compensate for these phase and baseline correction errors.

As next step those parameter ranges must be determined which definitively yield wrong measuring results or vice versa, the parameter sets required for accurate and precise measurements must be found and fixed. The criterion for robust parameters (in the following standard parameters) is that only a deviation of less 1% relative to the gravimetric reference value and a uncertainty of less than 1% is allowed. Table 3 summarizes all examined parameters and their variations. Parameter values with significant influences on accuracy are shown in italics, standard values are marked bold.

By varying the spectrometer parameters sets three different effects can be distinguished: (i) no significant influence (robust), (ii) significant influence on the signal-to-noise ratio, and (iii) systematic change of correct signal intensity.

Most of the parameters do not have significant effects on the accuracy or precision of the method within their evalu-

Table 3
Summary of all examined parameters and their variation ranges

	Acquisition parameters	Processing parameters	Evaluation parameters
Robust within examined range	Pulse power for 90° pulses in dB: **2**; 10 Preacquisition delay in μs: **5**, 10, 20, 50 and 100 Receiver gain: **512** (automatic), 256, 128 and 64 Sample temperature (K): **298** and 323	Zero filling: SI = TD; **2xTD**; 4xTD	Frequency-independent phase correction (°): 0, 0.05, 0.5, 1 and 2.5
Effect on signal-to-noise-ratio	Pulse angle (°): 90, 63, 45, **30**, 27, 18 and 9 Number of scans: 2048, 512, 256, 128, **32**, 16 and 8	Exponential multiplication (em) in Hz: **0.3**, 0.6 and 1.0	
Effect on signal intensities	Acquisition time (s): 0.1, 0.2, 0.4, 0.9, 1.7, **3.4** and 6.8 Relaxation delay (s): 30, 25, 20, 16, 12, 8, 5 and 3 (standard value 10 s for 30°pulse)		Frequency-dependent phase correction (°): 0, 0.2, 0.5, 2, 5 and 10

Bold values represent standard parameter sets, italics denote significant influences on the accuracy.

ated ranges (first case). For example, a change of pulse power with keeping the 90° flip angle constant does not cause a problem for ^{1}H NMR with a shift range of about 15 ppm. In the same way, a variation of the preacquisition delay from 5 up to 100 μs has proven to be robust for the samples studied here. In case that all resonance lines have similar line widths, the values of the preacquisition delay in the investigated limits is not important. It should be noted, that if very short delay between 5 μs and about 30 μs are used, the first data point is modified by the pulse ringdown due to the high Q-factor of the probes. As only the first data point is affected the Fourier transform causes minor distortions of the baseline; but these are not significant because the NMR lines are by orders of magnitude narrower and the baseline has to be corrected carefully around the resonance position anyway. The reason for choosing a short preacquisition delay is that if NMR lines with different transverse relaxation times occur (e.g. NH-protons), a longer preacquisition delay will change the relative peak areas and leads to systematic errors for the intensity ratios [30]. Furthermore, an increase of sample temperature did not change the results for this sample. Even drastic changes of the receiver gain (however, without overload of the ADC) do not change the results because of the long of word length of the ADC's.

Another parameter may be seen in the amount of zero filling. There is again no influence, provided that the FID has been measured long enough (see below). A criterion for this statement is that after the Fourier transform no significant wiggles appear. In Refs. [41–43] it is described, that at least five data points must appear above the half width for each resonance for a precise and reliable subsequent integration.

For the second group (ii) the signal-to-noise ratio (S/N) in the spectra can be influenced by variations of the pulse flip angle, the number of scans, and the line broadening (lb) using an exponential multiplication (em). The main result is that the given S/N for each signal affects strongly the precision. A detailed investigation shows (Fig. 4) that a S/N of at least 150 is required for the target uncertainty of 1%. A smaller flip angle decreases the S/N for a given experiment time and repetition delay. For short pulse angles (ca. 30° and less) the repetition time between the experiments can be shortened such that a better S/N can be obtained in the same experiment time (so-called Ernst angle [44]). Similarly, a larger line broadening improves the S/N, but the simultaneous line broadening of the signal may complicate the integration routine if an adjacent resonance is close. Following the suggestion of Günther [24], the best compromise is to use a small line broadening but a larger number of scans to increase the S/N ratio.

For group (iii) parameters like length of acquisition time, length of the relaxation delay and the frequency dependent (linear) phase correction of all lines. It has been found that an optimum exists for the acquisition time with respect to the best accuracy (here 3.4 s for sample NMR-1). Shorter and longer times decrease the S/N as a result of the acquisition of less signal intensity and of more noise, respectively. A lower S/N decreases clearly the precision. For rather short times (here lower than 0.9 s) the accuracy is influenced because the NMR signal is clipped. If no FID baseline correction is applied the Fourier transform produces broader signal forms with (substantial) 'wiggles' in the spectra. With FID baseline correction wrong intensities follows. The same effect was described by Rabenstein et al. [45]. The optimum acquisition time depends clearly on the line width, i.e. on the resolution of NMR spectra. Narrower signals (lower FWHH values) require longer FIDs. The relaxation delay is another important parameter. The longest T_1 relaxation time was 4.2 s such the relaxation delay must be set to at least 20 s (in case of a 90° pulse angle) in order to avoid selective saturation effects. Finally, frequency dependent phase errors of larger than 10° resulting from improper spectra processing cannot be corrected with the BIAS and SLOPE functions of the integration routine and yield significant deviations of the calculated areas of larger than 1%. This result agrees again with Rabenstein and Keire [29]. However, as such phase errors can be seen easily in the spectra they can be avoided.

For further investigations on comparability and for the continuation of the intercomparison program, two new model mixtures, NMR-2 and CCQM-P3, were prepared gravimetrically. Fig. 5 displays the NMR spectra of the aliphatic proton signals of sample NMR-2.

Based on the previous results a protocol for both the measurement and evaluation procedure of quantitative ^{1}H-SP-NMR was established. For this intercomparison the standard

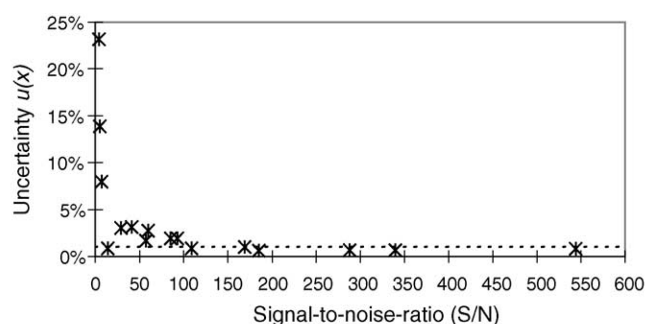

Fig. 4. S/N and its influence to the uncertainty of qNMR. Dotted line represents an uncertainty level of 1% relative.

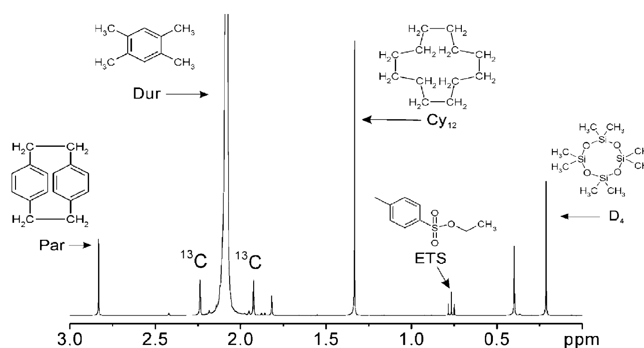

Fig. 5. ^{1}H-NMR of the model systems NMR-2. Aliphatic proton signals. Spectrum of CCQM-P3 is similar.

Table 4
Comparison of the results using various evaluation software packages: Bruker XWinNMR (XWN), Bruker WinFit (WF), and MestRe-C (MR) in different phase and baseline correction modes

		XWN-1	XWN-2	XWN-3	MR-1	MR-2	MR-3	WF
Correction	Phase	Man.	Man.	Auto.	Man (0.1°)	Man. (1°)	Auto.	By XWN
	Baseline	Man.	Man.	Auto.	Man.	Man.	Auto.	By XWN
	Integration	No	Man.	No	No	No	No	Not appl.
Compound ETS	Rel. Δx (%)	−0.1	−0.2	−3.9	−0.02	−4.0	−22	−6.3
	Rel. $u(x)$ (%)	0.4	0.4	0.24	0.6	3	11	1.4

Man. = manual; auto. = automatic; not appl. = not applicable.

parameter set of Table 1 were given to each participating laboratory. Furthermore, the relaxation delay was increased to 20 s to minimize selective saturation effects. This corresponds to seven times the longest relaxation time T_1 in the samples. All measurements were done without sample rotation in order to avoid the appearance rotational lines. Careful manual phase and baseline corrections have been prescribed, and, if needed, correction of the integral trials with BIAS and SLOPE were allowed. Furthermore, the integration limits for the NMR lines were correlated to the FWHH of each signal. The limits extend over a frequency range of 64 times the corresponding FWHH value ensuring that 99% of the whole signal is included [29].

The results of measurements at different magnetic field strengths B_0 and with different types of NMR probes proved the robustness of the method. If the same operator uses different NMR spectrometers (e.g. 400 MHz (BAM), 500 MHz, and 800 MHz (Bruker) with the sample NMR-2; 400 MHz (BAM), 300 MHz, and 600 MHz (Humboldt University) with solution CCQM-P3) a relative uncertainty value and a deviation from the reference values of smaller than 1% is obtained. If spectrometers are used that do not have digital filtering capability it must be ensured that the analog filter width is set properly. Otherwise intensity distortion may occur. It was found (using a 600 MHz spectrometer) that if the frequency offset of a resonance line from the r.f. is 3 kHz a filter width of 8.4 kHz causes an unacceptable signal damping of about 2.1%. The comparison of the performance of various NMR probes showed that the 10 mm probes (10 mm HR, 10 mm Si-special) lead to significant deviations up to 9% from the gravimetric reference values whereas the 5 mm qnp and txi probe at 400 MHz enable precise and accurate measurements. The reason is the larger inhomogeneous r.f. field in the 10 mm probe. Another problem was found for the 5 mm txi-cryo at 500 MHz (Bruker) which gave in general reliable results. However, for very intense signal strengths (here the main component Dur of NMR-2) radiation damping occurs obviously which causes a line broadening of this signal [46,47]. As a result the integration limits could be chosen only as 28 times the FWHH instead of 64. Hence, its intensity differs by 1.4% from the gravimetric reference value.

Next, the possible influences of various spectra processing and evaluation software packages have been tested (Bruker: XWinNMR (XWN), WinFit (WF), and the freeware MestRe-C (MR) [48]). Additionally, the manual correction of phase and the baseline modes were cross-checked with the automatic modes (XWinNMR, MestRe-C). The results for different combinations of correction modes are summarised in Table 4.

One of the main results is that precise and accurate results can be obtained only by an extremely careful manual spectrum correction and manual integration. The automatic correction routines of XWinNMR) work reliable, but for weak signals the integrals yield wrong values (too small) because it turned out, that baseline is slightly negative in such cases (ETS signal in NMR-2). The automatic routine of MestRe-C produces slightly wrong phase and baseline corrections. This yields inaccurate results with regard to the precision (relative uncertainty up to 11%) and the accuracy (relative deviation up to 22%).

Instead of the signal integration a line shape fit can be used to determine the signal areas of interest. The problem is that a unique profile for the line shape does not exist, because it depends – among others – on the shim stage (field homogeneity) of the magnets and including the probes (cf. Augner [49]).

Based on these robustness investigations and taking into account all the described problems an operation instruction was developed as a first step towards a norm for quantitative ^{1}H-SP-NMR. This instruction permits to obtain reliable, precise and accurate results, independent of the individual spectrometer configurations. Table 5 summarizes the specific spectrometer parameters. Furthermore, baseline and phase

Table 5
Summary of the universal spectrometer parameters for qNMR

Parameter	Nomenclature of Bruker	Value
90° pulse strength	Pl1	Instrument specific
90° pulse length	P1	Instrument specific
Spin rotation		Optional
Measurement temperature	TE	300 K
Frequency of excitation	o1	Middle of spectrum
Pulse angle		30°
Preacquisition delay	DE	5 μs
Acquisition time	AQ	3.41 s
Relaxation delay	D1	≥(7/3)x longest T_1
Sweep width	SW	16 ppm
Filter width	FW	≥20 ppm
Number of FID-points	TD	32 k
Number of scans	ns	Declined of reached S/N
Signal-to-noise ratio	S/N	≥150
Line broadening (em)	lb	0.3 Hz
Number of frequency-points	SI	64 k

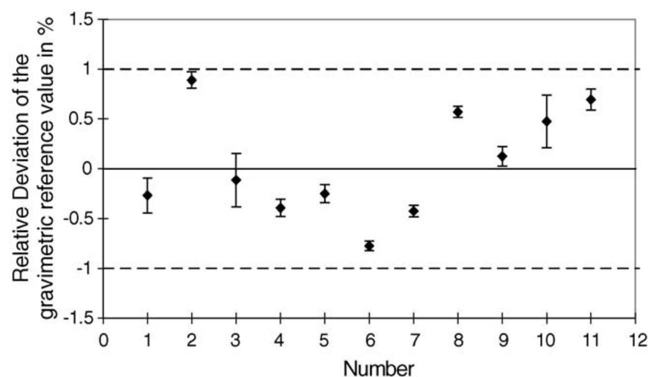

Fig. 6. Repeatability and comparability study at NMR-1 (ETS). Numbers 1–7 repeatable measurements over the time, 8 and 9 comparable measurements with another probe (txi) as well as 10 and 11 with another operator.

correction must be performed with high precision manually and correction of the integration trail is allowed. The integration limits cover a frequency range of 64 times the FWHH of the resonance line.

4.3. Parameters of accuracy

The robustness investigations give a first hint on the uncertainty for the repeatability and comparability. If a measurement is repeated five times with the universal parameter listed in Table 5 an extended uncertainty of 1.5% and less is obtained for each compound of the three model systems (NMR-1, NMR-2, CCQM-P3). Systematic errors (cf. Eq. (12)) were not found. Furthermore, eleven measurement series for the sample NMR-1 were carried out over a period of five months in order to estimate the value of repeatability as function of time. Also, the comparability was tested by changing both the NMR probe as well as the operator (Fig. 6). For each of these measurement series an extended uncertainty of less than 1.5% was obtained.

The same measurement uncertainty was obtained for spectrometers operating at various magnetic fields and different probes (cf. above).

Altogether, it was shown that a measurement uncertainty for qNMR of 1.5% can be achieved for a confidence interval of 95% ($k = 2$), which applies for the determination of molar ratios and of amount fractions.

4.4. Specificity and selectivity

As key prerequisites the specificity and selectivity must be checked for each sample prior to qNMR investigations. Specificity means the ability to assess unequivocally the analyte of interest in presence of other components (here for NMR: the unambiguous assignment of all NMR lines to the structure of the analyte). The selectivity of a method is given by the ability to determine analytes of interest in a complex mixture without interference from other components in the mixture. These checks have been carried out for all samples.

4.5. Intercomparison

To generalize the former results, another national intercomparison (NMR-2) was organized and performed. 33 laboratories of universities, research institutes and companies took part. Spectrometers of different manufacturers (Bruker, Varian, and Jeol) and with different B_0 field strengths (^{1}H NMR frequencies between 200 and 600 MHz) were used. All participants were asked to analyse the sample NMR-2 using the developed protocol (s. above, parameter values of Table 5) and the suggested careful manual phase and baseline corrections together with a manual integration procedure with an integration range of 64 times the FWHH.

Under these circumstances, most results of the molar ratio determination of all components (each referred to Cy_{12}) are within a deviation of less than 2% from the gravimetric reference values (numbers 12 and 14 are the BAM data). Fig. 7 shows the data for the component Par.

A similar result was obtained for the determination of the amount fractions. As seen in Fig. 7 few data show up with considerably larger deviations from the gravimetric reference value. It could be verified that these deviations were caused by the operators of participating laboratories. In some cases (numbers 35 and 36) improper phase and baseline corrections were applied or differently large integration limits were used (factors of 5 to 92 instead of 64). These results are not considered for this reason for the intercomparison evaluation.

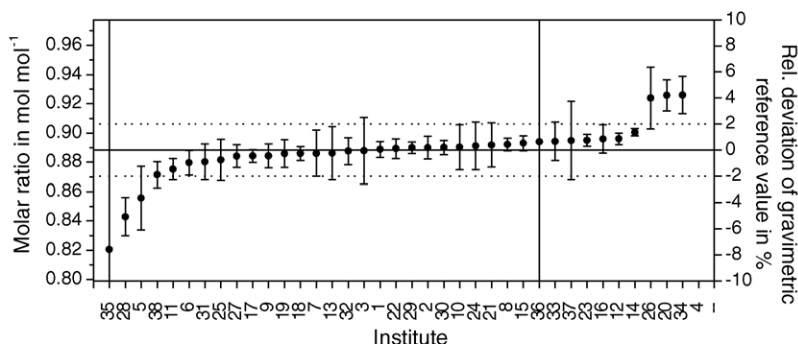

Fig. 7. Results of intercomparison NMR-2: molar ratio of Par refers to Cy_{12}.

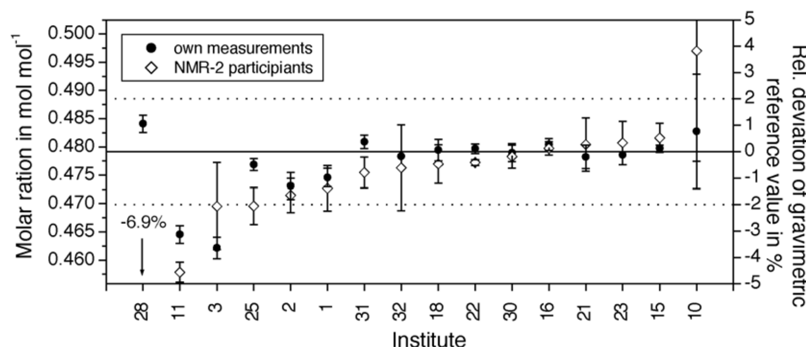

Fig. 8. Comparison of processing and evaluating by different operators.

It turns out, that the measurement uncertainty of 1.5% is obtained for a confidence interval of 95% ($k = 2$) for each compound, valid for determination of both the molar ratios as well as the amount fractions. This result demonstrates the importance of a protocol for accurate and precise measurement results with qNMR in comparison to the results of the two round robin test NMR-1 [21] and CCQM-4 [22] (measured without a protocol as described in chapter 1), where the results did differ enormously (up to 100%).

As next step, the raw data (NMR FID's) of 16 participants have been processed and evaluated by one of us (F.M.). The results are summarized Fig. 8. The general trend is that the precision is distinctly better under these circumstances. The strong influence of the 'quality' of the operator on the final results is obvious.

As a conclusion, qNMR can be used as analytical tool with a high metrological quality (cf. also Wells et al. [10,11] and Maniara et al. [20]). However, the results of the presented round robin tests proof the enormous influence of the operator on the accuracy.

In summary, a protocol has been developed that allows qNMR with a measurement uncertainty of 1.5% for $k = 2$, using the guidelines GUM [38] and EURACHEM [39]. This measurement protocol, summarized in Table 4, can serve as a standard operation procedure (SOP).

5. Results of purity determination of pharmaceutical substances

The purity determination of technical products uses the same principles as the molar ratio determination protocol. Therefore, the results of the robustness tests can be adopted.

In the following, the complete measurements procedure is described for cineole dissolved in DMSO-d_6. As pointed out earlier, the checks of the specificity and selectivity are the key issues because such samples are very likely to contain impurities.

The specificity test could be checked successfully. The recorded ^{1}H-NMR spectrum of the analyte (Fig. 9) can be assigned to cineole. 2D NMR (H,H-COSY, HMQC) support this assignment.

The investigation of selectivity was more difficult. The first point is, that the ^{1}H spectrum of the solvent showed the presence of impurities. These impurity signals are not superimposed with the cineole lines. However, impurity signals (marked by circles in Fig. 10) appear in the 2D H,H-COSY spectrum which are located directly under the analyte signal at 1.3–1.6 ppm (multiplet of protons 2,3a,4,5a and 6, numeration cf. Fig. 9). These impurity signals were recognized by cross peaks to other signals (two doublets at about 0.9 ppm) that have nothing in common with the cineole spectrum.

Dimethyl terephthalate was used as internal standard for the quantification. Its purity was traced back to the certified reference material benzoic acid (SRM 350a) by additional qNMR measurements. All experiments were carried with the parameters given in Table 5. The only exceptions concern a longer relaxation delay of 30 s (because of longer T_1 relaxation times of the aromatic protons of the standard) and the integrations limits for which only 32 times the FWHH was used. Sixty-four scans were accumulated. The analyte signal at 1.12 ppm was selected for quantification. It was evaluated against the monitor signal of the internal standard at 8.07 ppm (Fig. 11).

The purity determination was done by five weighings of about 10 mg of analyte and standard, solved in DMSO-d_6. The stability of such a solution was tested over three days prior the purity measurements. The calculated mean value

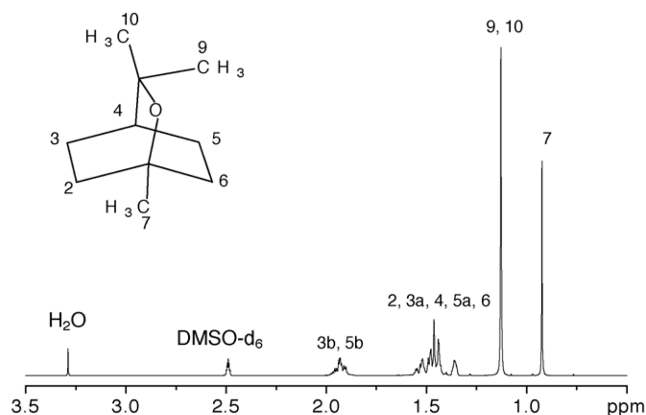

Fig. 9. Structure and ^{1}H-NMR spectrum of cineole.

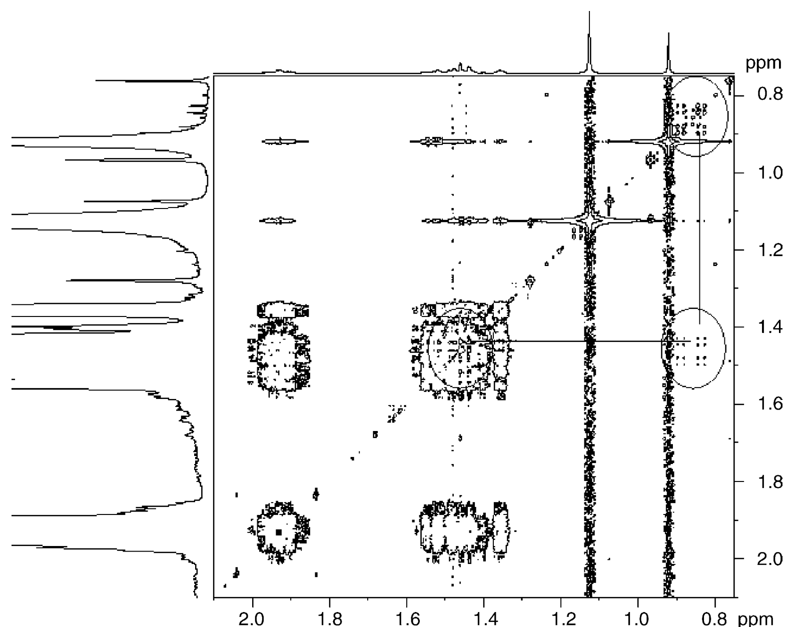

Fig. 10. COSY spectrum of cineole.

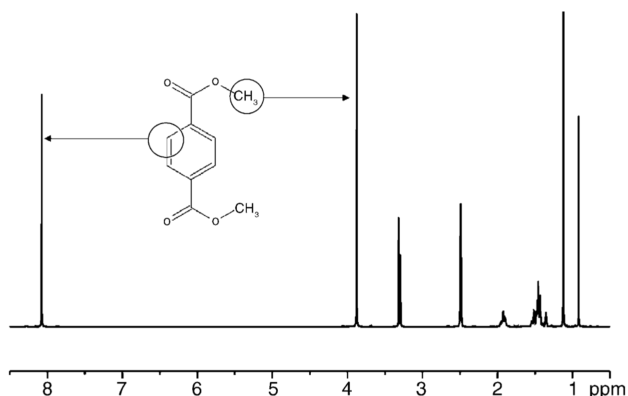

Fig. 11. Purity determination of cineole against internal standard dimethyl terephthalate.

of 98.8 g/g% with a variance of 0.1 g/g% was in good agreement with the reference value of the manufacturer, 98.5 g/g%, determined by gas chromatography (GC) and Karl-Fisher-titration (KF). The extended measurement uncertainty is 0.9 g/g% (eq. 8, $k=2$). Table 6 illustrates the complete uncertainty budget for this purity determination with qNMR.

Table 6
Uncertainty budget for the purity determination of cineole

Uncertainty	Value	$u(x)$	Rel. $u(x)$ (%)
Integration	98.81%	0.14%	0.14
Mol mass analyte (g mol^{-1})	154.24	0.008	0.005
Mol mass standard (g mol^{-1})	194.19	0.008	0.004
Weigh analyte (mg)	10.94	0.03	0.27
Weigh standard (mg)	10.06	0.03	0.30
Purity of standard	100.00%	0.15%	0.15
Comb. uncertainty			0.45
		0.45 g/g%	
		0.9 g/g%	

Last but no least, the linearity was verified once more using five solutions of different relative concentrations. A correlation coefficient of $r^2=0.9998$ was obtained. Further investigations are in progress for purity determinations of cavaine, cichoric acid, and rutin.

6. Conclusion

It has been shown that single pulse ^{1}H-NMR fulfils all requirements to be used as a validated method for quantitative determinations of amount fractions and molar ratios of dissolved sample mixtures. An operation protocol for quantitative ^{1}H NMR measurements has been developed that takes into account all relevant parameters for the data acquisition and data processing with subsequent evaluation. This protocol has been tested in a round robin test. This intercomparison also showed the significant influence of the operators handling the data. A main result is that qNMR can be preformed only with experienced personnel.

Following the suggested protocol a measurement uncertainty (calculated according to the guidelines of GUM and EURACHEM) of 1.5% is obtained (at a 95% confidence interval with $k=2$) for the determination of the molar ratios and the amount fractions. Finally, this procedure can also be used for validated purity determinations of pharmaceutical standards. A first example (cineole) is presented in this paper. Advantages of qNMR are a simple sample preparation and a rather quick and easy analysis (main component method).

Acknowledgments

Financial support by the Federal Ministry of Economics and Labour (BMWA) (AiF project: 13843 N/1), the German Federation of Industrial Cooperative Research Associations 'Otto von Guericke' (AiF) and the German Research Association of Medicine Manufacturers (FAH) is gratefully acknowledged. We thank Bruker, Rheinstetten, and the Humboldt University, Berlin, for using their spectrometers. It is a real pleasure to thank all participants of the intercomparisons.

References

[1] J.L. Jungnickel, J.W. Forbes, Anal. Chem. 35 (1963) 938–942.
[2] D.P. Hollis, Anal. Chem. 35 (1963) 1682–1684.
[3] J.W. Turczan, T. Medwick, Anal. Lett. 10 (1977) 581–590.
[4] A.F. Cockerill, G.L.O. Davis, R.G. Harrison, D.M. Rackham, Org. Magn. Reson. 6 (1974) 669.
[5] U. Holzgrabe, B.W. Diehl, I. Wawer, J. Pharmaceut. Biomed. 17 (1998) 557–616.
[6] A. Vailaya, T. Wang, Y. Chen, M. Huffman, J. Pharmaceut. Biomed. 25 (2001) 577–588.
[7] J. Forshed, F.O. Andersson, S.P. Jacobsson, J. Pharmaceut. Biomed. 29 (2002) 495–505.
[8] S. Meshitsuka, Y. Morio, H. Nagashima, R. Teshima, Clin. Chim. Acta 312 (2001) 25–30.
[9] U. Sahrbacher, A. Pehlke-Rimpf, G. Rohr, W. Eggert-Kruse, H.R. Kalbitzer, J. Pharmaceut. Biomed. 28 (2002) 827–840.
[10] R.J. Wells, J. Cheung, The Chemistry Preprint Server CPS: analchem/0103002 (2001).
[11] R.J. Wells, J.M. Hook, T.S. Al-Deen, D.B. Hibbert, J. Agric. Food Chem. 50 (2002) 3366–3374.
[12] Z. Xia, L.G. Akim, D.S. Argyropoulos, J. Agric. Food Chem. 49 (2001) 3573–3578.
[13] T. Igarashi, M. Aursand, Y. Hirata, I.S. Gribbestad, S. Wada, M. Nonaka, JAOCS 77 (2000) 737–748.
[14] N. Papke, J. Karger-Kocsis, Eur. Polym. J. 37 (2001) 547–557.
[15] T.J. Henderson, Anal. Chem. 74 (2002) 191–198.
[16] H. Jancke, Nachr. Chem. Tech. Lab. 46 (1998) 720–722.
[17] B. King, Accredit. Qual. Assur. 5 (2000) 429–436.
[18] B. King, Accredit. Qual. Assur. 5 (2000) 266–271.
[19] M.J. Milton, T.J. Quinn, Metrologia 38 (2001) 289–296.
[20] G. Maniara, K. Rajamoorthi, S. Rajan, G.W. Stokton, Anal. Chem. 70 (1998) 4921–4928.
[21] H. Jancke, F. Malz, Abschlussbericht NMR-1, Berlin, 1999.
[22] H. Jancke, F. Malz, International Comparison CCQM-4. Final Report, Berlin, 1999.
[23] A.E. Derome, Modern NMR Techniques for Chemistry Research, Pergamon Press, Oxford, 1987.
[24] H. Günther, NMR Spectroscopy, Wiley, Chichester, 1995.
[25] H. Friebolin, Basic One-Dimensional and Two-Dimensional NMR Spectroscopy, Wiley-VCH, Weinheim, 1998.
[26] M.H. Levitt, Spin Dynamics: Basics of Nuclear Magnetic Resonance, Wiley-VCH, Chichester, 2001.
[27] R. Freeman, Handbook of Nuclear Magnetic Resonance, 2nd ed., Addison Wesley Longman, Edinburgh, 1997.
[28] M.L. Martin, G.J. Martin, J.-J. Delpeuch, Practical NMR Spectroscopy: Practical Aspects of Intensity Measurement and Quantitative Analysis, Heyden & Sons, London, 1980 (Chapter 9).
[29] D.L. Rabenstein, D.A. Keire, Pract. Spectrosc. 11 (1991) 323–369.
[30] C. Szantay Jr., Trends Anal. Chem. 11 (1992) 332–344.
[31] L. Griffiths, A.M. Irving, Analyst 123 (1998) 1061–1068.
[32] C.K. Larive, D. Jayawickrama, L. Orfi, Appl. Spectrosc. 51 (1997) 1531–1536.
[33] S. Kromidas, Validierung in der Analytik, Wiley-VCH, Wienheim, 1999.
[34] ISO/IEC Guide 25, General requirements for the competence of calibration and testing laboratories, Geneve, 1990.
[35] DIN EN 45001, General requirements fort he competence of testing and calibration laboratories, Geneve, 1997.
[36] EN ISO/IEC 17025, General requirements fort he competence of testing and calibration laboratories, Geneve, 1999.
[37] S. Kromidas, Nachr. Chem. Tech. Lab. 46 (1998) 28–32.
[38] BIPM, Guide to the Expression of Uncertainty Measurement, 2nd ed., International Organization for Standardization, Geneva, Switzerland, 1995.
[39] EURACHEM, Quantifying Uncertainty in Analytical Measurements, 2nd ed., London, 2000.
[40] S. Noack, W. Hässelbarth, Konzept zur Ermittlung und Angabe von Messunsicherheiten in der Abteilung Analytische Chemie; Referenzmaterialien, Federal Institute for Material Searching and Testing (BAM), Berlin, 2002.
[41] F.G. Herring, P.S. Phillips, J. Magn. Reson. 62 (1985) 19.
[42] J.-P. Grivet, Signal Treatment and Signal Analysis in NMR: Accuracy and Precision of Intensity Determinations in Quantitative NMR, Elsevier, Amsterdam, 1996 (Chapter 14).
[43] K. McLeod, M.B. Comisarow, J. Magn. Reson. 84 (1989) 490–500.
[44] R.R. Ernst, W.A. Anderson, Rev. Sci. Instrum. 37 (1966) 93–102.
[45] D.L. Rabenstein, K.K. Millis, E.J. Strauss, Anal. Chem. 60 (1988) 1380A–1391A.
[46] C.R. Bruce, R.E. Norberg, R.V. Pake, Phys. Rev. 104 (1956) 419–420.
[47] S. Bloom, J. Appl. Phys. 28 (1957) 800–805.
[48] C. Cobas, J. Cruces, F.J. Sardina, MestRe-C 2.1, Universidad de Santiago de Compostela, http://qobrue.usc.es/jsgroup/mestre-c/mestre-c.html.
[49] S. Augner, Beiträge zur quantitativen Analytik mit Hilfe von spektroskopischen Methoden. Degree dissertation, Heinrich-Heine-Universität, Düsseldorf 1999.