

Quantitative NMR for Content Assignment of Reference Standards

For reference standards used as primary standards for pharmaceutical quality control, stability testing and in-process control of herbal medicinal products and respective starting materials a dossier together with a certificate of analysis must be available. In this dossier information on identity testing (e.g. by NMR, MS, IR, UV), purity testing (e.g. water content, residual solvents, inorganic impurities) and the assigned content of the reference standard must be provided.

Content assignment by chromatographic methods and purity testing

For content assignment assay is often performed using two methods preferably orthogonal or at least independent from each other. For this purpose chromatographic methods are usually applied. Content assignment is then performed based on the results obtained from purity testing and the respective area percentage of the reference standard in the chromatographic separation systems used. This process ignores the fact that such a procedure only provides sufficiently accurate results if the purity of the reference standard is high, i.e. above 99.5%. Furthermore the uncertainty of the content assigned on the basis of results obtained with 3 – 4 analytical methods using the following equation is quite high:

$$C = \frac{CP \times (100 - RS - W - I)}{100}$$

Where C = assigned content, CP = chromatographic purity, RS = residual solvents, W = water content, I = inorganic impurities

Reference standards obtained from natural sources may have a complex molecular structure, which is why they are hardly accessible synthetically. When isolating the substances from plants, however, a purity exceeding 99.5% can only be achieved with considerable effort. In addition, within the scope of isolation, purification is often performed via chromatographic techniques, thus usually applying the same methods as for content and purity analysis within the framework of the primary standard establishment. This results in the risk that impurities that could not be separated during purification may also be overlooked during the establishment of the standard. Such impurities are frequently biogenetically related to the target molecule and display very similar structures, which makes analytical separation even more difficult. The selectivity (or specificity) of the method applied for purity testing hence plays a central role, especially with respect to reference standards isolated and purified from natural sources. As a result, the content of reference standards isolated from natural sources may be difficult to be assigned by using this established procedure.

Content assignment by quantitative NMR

Avoiding all problems described, „direct“ or „relative“ primary methods of measurement conveying a direct traceability to the SI units ensure an essentially higher metrological quality [1]. These methods are increasingly applied for content assignment of natural products used as pharmaceutical reference standards.

Quantitative NMR spectroscopy (qNMR) is a potential relative primary method [2]. Employment of qNMR

would essentially simplify and increase the reliability of the establishment of reference standards and their certification. Its basic principle is well known and described in a series of monographs [3-10]. The most important basis of quantitative NMR spectroscopy is the direct proportionality of the signal intensity to the number of nuclei contributing to the resonance line.

In general there are two possibilities of content assignment, that of direct analysis via the principal component and that of indirect analysis via the impurities. Since content assignment plays a central role in the qualification of reference standards, the direct method is usually applied.

In doing so the principal component (analyte), i. e. the reference standard, is evaluated against an internal standard. The advantage of this method lies in the fact that only the unequivocal assignment of one undisturbed signal of the principal component is required. In this context knowledge about the composition and/or qualitative assignment of the other signals is usually not necessary.

For determination of the content of the principal component, an internal standard and the principal component have to be weighed into one NMR tube together (m_x , m_{Std}). The intensities of appropriate signals of the principal component (I_x) and the internal standard (I_{Std}) are used for calculation. Based on the ratio of intensities, considering the number of nuclei contributing to the resonance (N_x , N_{Std}), the molar masses (M_x , M_{Std}) and initial weight of internal standard (considering its relative content P_{Std}) and analyte, the relative content of the principal component (P_x) can be calculated as w/w % using the following equation:

$$P_x = \frac{I_x}{I_{Std}} \cdot \frac{N_{Std}}{N_x} \cdot \frac{M_x}{M_{Std}} \cdot \frac{m_{Std}}{m_x} P_{Std}$$

Additionally not only content assignment could be performed using the method described but also in one step the proof of identity using the NMR method in the classical way for structural analysis. Hence the substance supply required for analysis, for instance the very expensive natural products, could essentially be decreased, thus saving costs.

The qualitative assignment of signals in the spectrum (specificity and selectivity) is the virtually most important part of the quantitative analysis. The larger and more complex the molecule, the more difficult becomes this evaluation. An additional aggravating factor is the fact that in case of complex compounds impurities with a very similar structure hardly differ at all in the spectrum and/or only in few places. This problem, however, is not NMR-specific but can be transferred to all spectroscopic and chromatographic methods.

Nevertheless, where other methods fail because the purity of the reference standard cannot be selectively established, qNMR provides accurate results by evaluation via further signals in the spectrum. Furthermore, for some substances additional impurities not detected using chromatographic methods can be detected and partly even identified and quantified by qNMR. For each target analyte selectivity of the signals used for analysis has got to be established by means of $^1\text{H-NMR}$ and correlated 2D NMR techniques (COSY, HMQC, HMBC). The data obtained can also be used for assignment of signals originating from impurities.

Method validation

The European CCQM (Comité Consultatif pour la Quantité de Matière) has the task of ensuring the harmonisation of physical parameters as well as accurately describing and improving the accuracy and precision of measurement methods in analytical chemistry. Within the efforts made by the CCQM, the German Federal Institute for Materials Research and Testing (BAM) has been charged with defining the capabilities of quantitative NMR spectroscopy and proving its effectiveness in international interlaboratory tests [11]. Comprehensive validation results for the certification of reference standards could be established within a government funded project in Germany, which are largely publicly available now [12]. Since NMR spectroscopy has the character of a primary method, as stated in papers for the CCQM [11, 12], all prerequisites are fulfilled to be able to perform metrologically top-quality, SI-based certifications for pharmaceutical reference materials, too. In Ph. Eur. the monograph on NMR spectroscopy was revised based on the results of the research project, now permitting the use of qNMR as an official method in pharmaceutical analysis.

During realisation of this project qNMR was proven to be appropriate as a potential primary analytical method for the qualification of natural products as reference standards for quality control of herbal medicinal products. Using a total of 14 samples of selected reference materials relevant for the analysis of herbal medicinal products (ginkgolide A, cichoric acid, chlorogenic acid, rutin trihydrate, pseudohypericin, hypericin, hyperforin, 1,8-cineole, ginsenoside Rc, alpha-onocerin, kavain), the metrological quality of the quantitative high-resolution ¹H-NMR and hence its suitability for pharmaceutical analysis were investigated.

As a result a standard operating procedure (SOP) is now available for the recording, processing and evaluation of qNMR measurements for the qualification of reference standards.

To confirm the acceptance of qNMR results by the national regulatory authority (BfArM) and the European EMEA, these studies were conducted according to the ICH guidelines [13]:

Assay and determination of precision based on six independent analyses (six initial weights) and verification of linearity based on five measuring points in relative contents of 70 %, 85 %, 100 %, 115 % und 130 %, with reference to the target content. Additionally further investigations on robustness with respect to solvents, quality of NMR tubes, analyst and time of analysis were exemplarily performed on kavain and rutin.

By means of an organised and evaluated national interlaboratory test with 22 participants from industry, research organisations and universities, qNMR was proven to be sufficiently reproducible.

In order to determine the metrological quality (accuracy and precision), the complete uncertainty budget according to ISO guidelines was established for content assignment of exemplary reference standards using qNMR spectroscopy. Generally an extended uncertainty of measurement ($k = 2$) of $\leq 1\text{g/g} \%$ was determined for all substances investigated.

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About HWI ANALYTIK GmbH

For nearly 20 years HWI has been a provider of services in pharmaceutical analysis and drug safety and a manufacturer of pharmaceutical reference standards. More than 15 years of experience in the isolation of marker compounds for testing herbal medicinal products and respective starting materials and in the subsequent establishment as primary and secondary reference standards have resulted in a comprehensive product portfolio and know-how.

The laboratory services comprise method development and validation, stability testing, impurity testing, dissolution testing and isolation/synthesis of marker compounds for herbal medicinal products and impurities. HWI establishes and qualifies reference standards for quality control, in-process control and stability testing. These reference standards are either offered as primary standards with comprehensive information on identity, content and purity or as working standards. The content of these working standards is derived by intercalibration against primary standards.

HWI's new product line of primary reference standards qualified by qNMR is exclusively distributed by Sigma-Aldrich.

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