

Application of Reference Standards in the Analysis of Herbal Medicinal Products

Gerrit Foerster, Head of Isolation Lab, HWI ANALYTIK GmbH, Rheinaberner Str. 8, 76761 Ruelzheim, Germany
 Frank Michel, Head of Marketing, HWI ANALYTIK GmbH, Rheinaberner Str. 8, 76761 Ruelzheim, Germany fmichel@hwi-analytik.de
 Matthias Nold, Product Manager Analytical Standards matthias.nold@sial.com



Figure 1 Hawthorn (*Crataegus monogyna*)

In collaboration with HWI ANALYTIK, Sigma-Aldrich is launching a new product line of primary reference standards for use in the quality control, in-process control and stability testing of herbal medicinal products.

These standards are produced and qualified by HWI ANALYTIK in Ruelzheim (Germany), and are exclusively distributed by Sigma-Aldrich. A first series of 23 standards is listed in **Table 1**. The unique and novel certification concept of these reference standards is based on the quantitative NMR technique (qNMR) for content assignment. The application of qNMR, a relative primary method of measurement, is advantageous compared to the usual combination of two chromatographic methods, Karl Fischer titration and residual solvent determination due to higher reliability of the certified content value. The analytical report delivered with these products contains two identity proofs, the content by qNMR and the chromatographic purity. Comprehensive documentation on further parameters, detailed method descriptions and validation data can be ordered on request directly from HWI ANALYTIK.

While the following article in this issue (page 14 f) focuses on the content assignment of these standards by quantitative NMR, this article addresses the applications of reference standards in the analysis of herbal medicinal products.

Reference standards used for pharmaceutical analysis in a GMP environment are standards for qualitative and/or quantitative determinations within the scope of in-process controls, batch release analyses and stability studies of herbal drugs, herbal preparations and finished products. In case of herbal medicinal products, the reference standard may be a standard specimen of the drug, the drug preparation (e. g. extract or tincture) or a chemically defined substance. Regarding the latter case, only a limited number of herbal substances and herbal preparations possess constituents which are generally accepted to contribute substantially to their therapeutic activity. These are defined as 'constituents with known therapeutic activity', e. g. sennosides in preparations from senna leaves or fruits. However, for the majority of herbal substances and herbal preparations, the constituents or groups of constituents responsible for the therapeutic activity are not yet known. In some cases, certain constituents or groups of constituents may be generally accepted to contribute to the therapeutic activity but are not responsible for the full therapeutic effect. Such constituents or groups of constituents are useful for control purposes and are defined as 'active markers'. Distinct from them, analytical markers are constituents or groups of constituents that serve solely for analytical purposes [1].

(continued on page 12)



Figure 2 Lemon balm (*Melissa officinalis*)

Markers are used for both quantitative and qualitative purposes. For all kinds of analytes – constituents with known therapeutic activity, active and analytical markers – there is a need to establish reference standards for quality control and stability testing of herbal preparations and herbal medicinal products.

In the pharmaceutical environment, a primary reference standard initially needs to be established and certified. Its content should be assigned without requiring comparison to another chemical substance. Primary reference standards in pharmaceutical analysis are of a defined purity. A comprehensive documentation needs to be filed for the marketing authorisation dossier. The process of establishment, certification and content of documentation to be filed has recently been described by Veit and Wissel [2, 3].

For routine analysis, working standards may be used. Working standards are secondary standards serving as ready-to-use reference standards for the testing. They are derived from primary reference standards by intercalibration. The extent of characterisation and testing of a secondary chemical reference substance is less than that of a primary chemical reference substance.

Great difficulties may arise when it comes to establishing natural product standards for use in identity testing and assays by the application of constituents with known therapeutic activities or (active) markers. Substances of natural origin usually display a complex molecular structure, which is why they are hardly accessible synthetically and quite frequently have to be isolated from the natural sources. Hence, establishing primary standards for the quality analysis of herbal medicinal products is often very complex and requires special know-how. Additional problems then arise due to the small amounts accessible this way, thus requiring an elaborate concept for the storage and employment of such standards. Special suppliers have therefore started to establish, store and manage the primary standards at a central site and supply their customers with qualified working standards that are ready to use.

Product Name	Prod. No.	Pack Size
Bisabolol oxide A, primary reference standard	00630590	25 mg
Chlorogenic acid, primary reference standard	00500590	10 mg
Coumarin, primary reference standard	01260595	25 mg
Ginkgolide A, primary reference standard	00770590	10 mg
* Ginkgolic acid C15:1, primary reference standard	02580185	10 mg
Ginkgolic acid C17:1, primary reference standard	01390590	10 mg
Ginsenoside Rb1, primary reference standard	00170580	10 mg
Ginsenoside Rg1, primary reference standard	00370580	10 mg
Harpagoside, primary reference standard	00420580	10 mg
Hulupinic acid, primary reference standard	01090595	10 mg
* Hypericin, primary reference standard	00190180	10 mg
Hyperoside, primary reference standard	00180585	10 mg
Isoquercitrin, primary reference standard	00140585	10 mg
Quercetin dihydrate, primary reference standard	00200595	50 mg
Quercitrin, primary reference standard	00740580	10 mg
Rosmarinic acid, primary reference standard	00390580	10 mg
Rutin trihydrate, primary reference standard	00300590	50 mg
Sennoside A, primary reference standard	01870575	10 mg
Sennoside B, primary reference standard	00530580	10 mg
Silibinin, primary reference standard	02000585	10 mg
Valerenic acid, primary reference standard	02010595	10 mg
Vitexin-2"-O-rhamnoside, primary reference standard	00660585	10 mg
Xanthohumol, primary reference standard	01130595	10 mg

Table 1 Primary Reference Standards from HWI ANALYTIK exclusively available at Sigma-Aldrich

* for Ginkgolic acid C15:1 and Hypericin, the content determination by qNMR was not applicable due to overlapping signals. For these two products, the content has been assigned by the usual mode combining two chromatographic methods, water content by Karl Fischer and determination of residual solvents and of inorganic compounds.

Batch-specific control of a herbal medicinal product containing extracts of lemon balm and hawthorn

In herbal medicinal products containing more than one herbal preparation, the choice of appropriate reference standards becomes far more challenging, because substances usually used as reference standards for a dedicated plant may occur in the other component of the combination drug, too. Due to this challenge, reference standards for a combination product should be selected in such a way that they are characteristic for each individual herbal preparation in the product. In the following example, the batch-specific control of a herbal medicinal product with the two main components, lemon balm and hawthorn, is presented.

For the quantification of hawthorn, the constituent vitexin-2''-O-rhamnoside is used as an analytical marker (chromatogram and the structure of the reference standard vitexin-2''-O-rhamnoside are shown in **Figure 3a**). The chromatogram of an extract of hawthorn (**Figure 3b**), due to its complexity, highlights the need for an appropriate reference standard in the analysis of herbal medicinal products.

One main component of the extract of lemon balm is rosmarinic acid, which is used as an active marker (a chromatogram and the structure of rosmarinic acid are shown in **Figure 3c**; a chromatogram of an extract of lemon balm is shown in **Figure 3d**).

In **Figure 3e**, the chromatogram of the final herbal medicinal product is presented. The content of hawthorn and lemon balm extract in the final medicinal product is determined by the use of the reference standards vitexin-2''-O-rhamnoside and rosmarinic acid. This chromatogram highlights the challenge of the analysis of herbal medicinal products due to their complex composition.

References:

- [1] European Medicines Agency (2008): Reflection paper on markers used for quantitative and qualitative analysis of herbal medicinal products and traditional herbal medicinal products. Available at: www.emea.europa.eu/pdfs/human/hmpc/25362907enfin.pdf (05.11.2009)
- [2] Veit, M.; Wissel, S. *PharmInd* 2007, 12, 1475–1480
Available in English at: www.hwi-analytik.de/en/download.html
Available in German at: www.hwi-analytik.de/de/download.html
- [3] Veit, M.; Wissel, S. *PharmInd* 2008, 1, 135–138
Available in English at: www.hwi-analytik.de/en/download.html
Available in German at: www.hwi-analytik.de/de/download.html

Chromatographic conditions for Fig. 3a-e:

HPLC: Alliance 2695, Waters
 Detector: DAD @ 330 nm for rosmarinic acid
 DAD @ 340 nm for vitexin-2''-O-rhamnoside
 Column: Synergi Polar RP; 250 x 4,6 mm (4 µm) with guard column
 Temperature: 25 °C
 Mobile Phase: Gradient: Water/TFA (pH 2,0)/acetonitrile
 Flow rate: 1,0 ml/min
 Injection volume: 20 µl

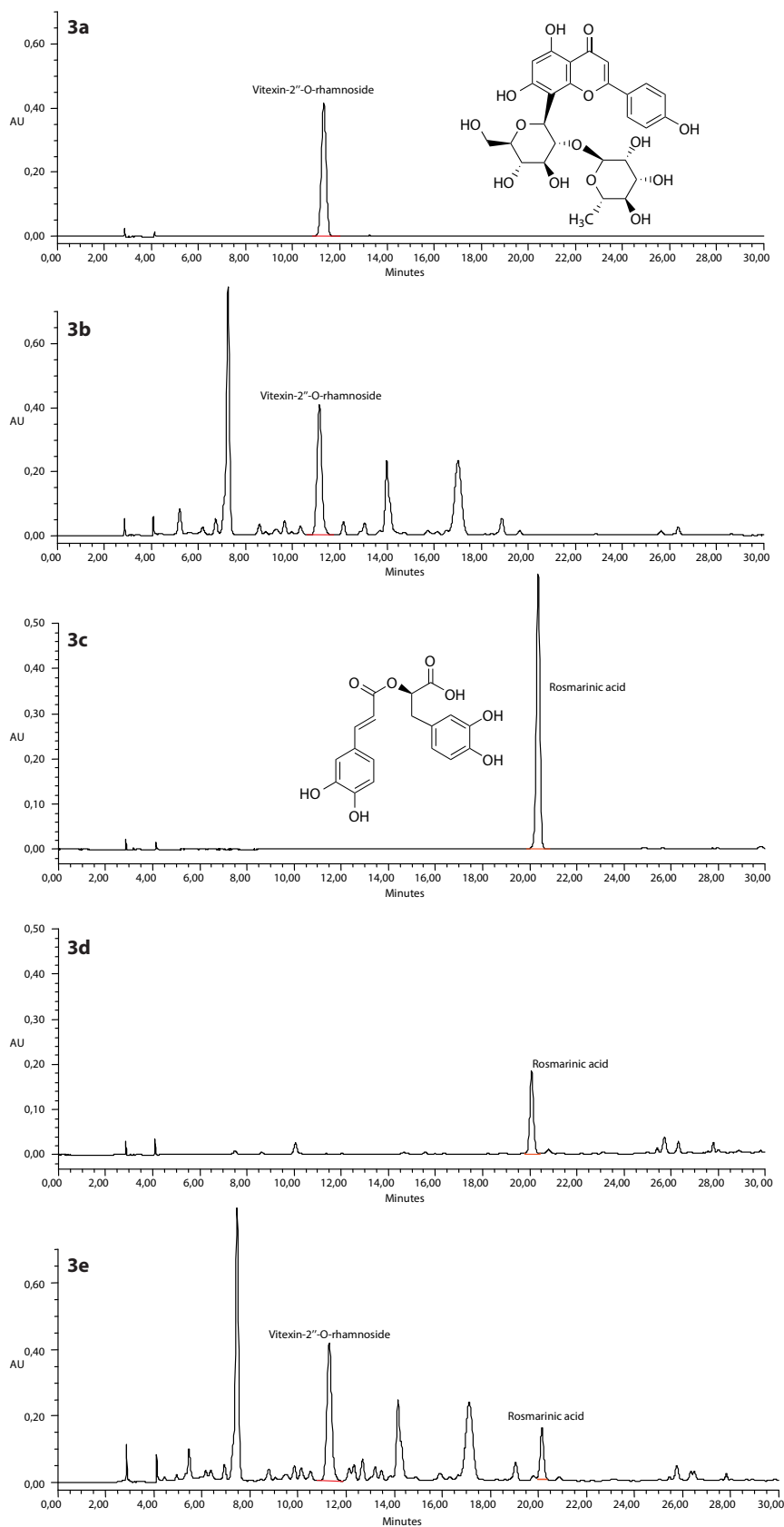


Figure 3a) Reference standard vitexin-2''-O-rhamnoside, $c = 0,15$ mg/ml; **3b)** Sample solution of an extract of hawthorn leaves, flowers and fruits; **3c)** Reference standard rosmarinic acid, $c = 0,1$ mg/ml; **3d)** Sample solution of an extract of lemon balm leaves; **3e)** Sample solution of the herbal medicinal product, exemplary recorded at 340 nm.

Quantitative NMR Used for Content Assignment of Reference Standards for Quality Control of Herbal Medicinal Products

Markus Veit, CEO, i.DRAS GmbH, Fraunhoferstrasse 18b, 82152 Planegg/Martinsried, Germany markus.veit@i-dras.com

Within the scope of a marketing authorisation procedure for herbal medicinal products, a comprehensive dossier and a certificate of analysis must be compiled for all reference standards used. In this dossier, information on identity (NMR, MS, IR, UV, and others), purity (water content, residual solvents, inorganic impurities, and organic impurities), and the content of the reference standard must be provided. Currently, assay is performed using two methods which preferably are independent from each other. For this purpose, chromatographic methods are usually applied. Content assignment is then performed based on the results obtained from the purity tests and the respective area percentage of the analyte in the chromatographic separation systems. However, this procedure is only applicable for sufficiently pure (>99.5%) reference standards.

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

Avoiding all problems described, "direct" or "relative" primary methods of measurement that convey a direct traceability to the SI units ensure a 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 simplifies and increases the reliability of the establishment of reference standards and their certification. The most important basis of quantitative NMR spectroscopy is the direct proportionality of the signal intensity with the number of nuclei contributing to the resonance line.

In general, there are two possibilities of content analysis: 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 certification of reference standards, the direct method is applied here. In doing

so, the principal component, 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 an optimally integrable 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, the internal standard and analyte must be weighed together into one NMR tube. Intensities of appropriate selective signals of the principal component and the internal standard are used for calculation. Based on the ratio of intensities, the relative content of the analyte can be calculated as m/m%.

As an example, the spectrum of vitexin-2"-O-rhamnoside containing 2-hydroxy-3,5-dinitro benzoic acid as an internal standard is shown in **Figure 1**.

Ultimately, not only content assignment could be performed using the method described, but also the proof of identity in one step using the NMR method in the classical way for structural analysis. Hence, the supplied substance requiring certification, for instance the very expensive natural products, could essentially be decreased, thus reducing the high costs associated with the extensive effort in connection with isolation and purification.

The European CCQM (Comité Consultatif pour la Quantité de Matière) ensures the harmonisation of physical parameters as well as accurately describing and improving the accuracy and precision of measurement methods in analytical chemistry. From CCQM directives, 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 [3]. In this respect, BAM acts as a national metrology institute for Germany. Within a public-funded project in Germany, comprehensive validation results for the certification of reference standards could be established, which are largely publicly available now [4]. Since NMR spectroscopy has the character of a primary method, as demonstrated in publications for the CCQM [3,4], all requirements are satisfied to perform metrologically top-quality, SI-based certifications for pharmaceutical reference materials. 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 could be proven to be basically appropriate as a potential primary analytical method for the certification 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, the metrological quality of the quantitative ¹H-NMR, and hence its suitability for pharmaceutical analysis, were investigated.

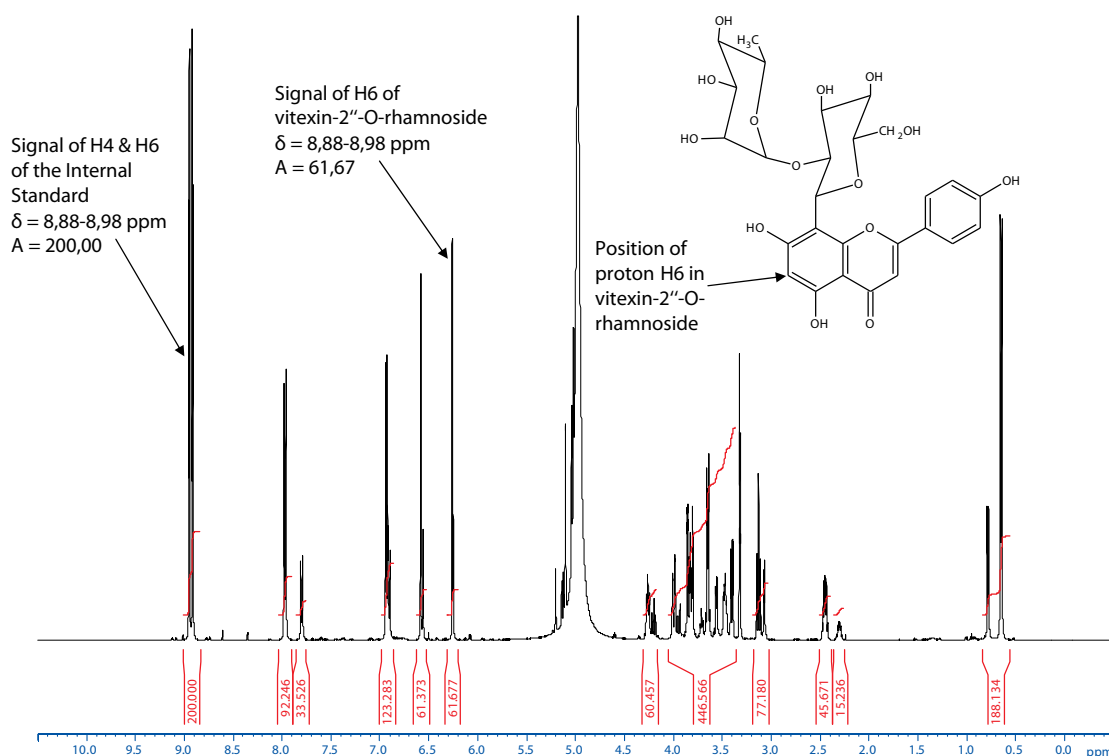


Figure 1 NMR spectrum of vitexin-2''-O-rhamnoside and the Internal Standard 2-hydroxy-3,5-dinitro benzoic acid. For quantification of the content of the principal component the signals of proton H6 of the vitexin-2''-O-rhamnoside and of the aromatic protons (H4 and H6) of the 2-hydroxy-3,5-dinitro benzoic acid are used.

The qualitative assignment of signals in the spectrum (specificity and selectivity) was shown to be the most important part of the quantitative analysis. The larger and more complex the molecule, the more difficult this evaluation became. Additionally aggravating was the fact that, in the case of complex compounds, impurities with a very similar structure differed just slightly in the spectrum and/or only at a few frequencies. This problem, however, is not NMR-specific but can be transferred to all spectroscopic and chromatographic methods. Nevertheless, where other methods fail because the analyte cannot be selectively quantified, qNMR provided accurate results in such cases by evaluation via further signals in the spectrum. Furthermore, for some substances, additional impurities not detected using chromatographic methods could be detected and even partially identified and quantified by qNMR. For each target analyte, selectivity of the signals used for analysis was established by means of ^1H -NMR (partly using ^{13}C decoupling) and ^1H , ^{13}C hetero correlated 2D NMR techniques (HMQC, HMBC), and for assignment of signals originating from impurities by H,H correlated 2D NMR techniques (COSY).

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

To confirm the acceptance of qNMR results by the German national regulatory authority (BfArM) and the European

EMA, these studies were conducted according to the ICH guidelines [5]. By means of an organised and evaluated national interlaboratory test with 22 participants from industry, research and university facilities, the generalisation of laboratory internal validation was proved unequivocally on the basis of one substance.

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

References

- [1] Malz, F. Quantitative NMR-Spektroskopie als Referenzverfahren in der analytischen Chemie. Dissertation Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin 2003.
- [2] Jancke, H. NMR als primäre Methode. *Nachrichten aus Chemie, Technik und Laboratorium*, 1998, 46, 722.
- [3] Jancke, H. NMR Spectroscopy as a Primary Analytical Method, Document 98/02 to the 4th Session of the CCQM, Sèvres 1998.
- [4] Malz, F.; Jancke, H. Validation of quantitative NMR. *Journal of Pharmaceutical and Biomedical Analysis* 2005, 38, 813–823.
- [5] ICH Q2R: Validation of analytical procedures. Available at: www.emea.europa.eu/pdfs/human/ich/038195en.pdf