Vietnam Journal of Science and Technology ***58*** *(6A) (2020) 158-166*

doi:10.15625/2525-2518/58/6A/15500

QUANTITATIVE NUCLEAR MAGNETIC RESONANCE BASED ON PULCON METHODOLOGY: APPLICATION TO QUANTIFICATION OF SOME STANDARD MATERIALS AND NATURAL PRODUCTS

Nguyen Quang Tam*, Dang Vu Luong, Do Thi Thanh Xuan, Thanh Thi Thu Thuy, Nguyen Tien Tai

Institute of Chemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Ha Noi, Viet Nam

*Email: nguyenquangtam@gmail.com

Received: 15 September 2020; Accepted for publication: 18 December 2020

Abstract. Currently, both internal standard quantitative nuclear magnetic resonance (IS qNMR) and external standard quantitative nuclear magnetic resonance (ES qNMR) methods are not widely disseminated in Viet Nam. In this report, the PULCON (Pulse Length-Based Concentration) based ES qNMR method was applied for quantification of two standard materials (2-acetylthiophene and 4-methylmorpholine) and three natural products (curcumin, quercetin and rutin). The obtained results using the PULCON based ES qNMR method were in reasonable agreement with the reference data from manufacturers and those obtained by IS qNMR method. The error is less than 1 % when compared to the result obtained by IS qNMR and manufacturer's data. In addition, the precision is relatively low (0.23 - 0.38 %), which equals to the precision of IS qNMR method. Thus, PULCON based ES qNMR method can be applied for routine quantitative analysis, especially for the expensive and new compounds.

Keywords: qNMR, PULCON, certified reference materials, chemical pharmaceutical materials.

Classification numbers: 1.1, 1.1.3, 1.1.6.

1. INTRODUCTION

NMR spectroscopy is a powerful technique for structural determination of organic compounds. As the NMR signal intensity is proportional to the number of corresponding nuclei and it does not depend on the structure of molecules, NMR spectroscopy has been also applied as a quantitative analytical technique [1, 2]. The quantitative NMR (qNMR) method is based on the comparison of one or more NMR signals of analytes with a signal of reference standard (RS), which is a compound of known concentration. To achieve this, RS is solubilized together with the analytes in the same NMR tube, forming a mixed sample. Such techniques are called internal standards (IS) qNMR, which has found widespread use in various areas of chemistry, biology, pharmacy and medicine [3 - 6].

Although IS qNMR method can yield high accuracy and precision, it has several drawbacks. Firstly, the sample can be contaminated by the RS and therefore recovery of the

sample for subsequent analytical or testing experiments can become problematic; secondly, the reference standard should be chosen so that it does not react with the analyte and there is no overlap of its NMR signal with that of the analyte nor the reduced water or solvent. These problems can be overcome using the external standard (ES) qNMR method, where the RS and the analyte are not mixed together but in separate NMR tubes.

In fact, there are several technical solutions for implementing ES qNMR method, of which PULCON (Pulse Length-Based Concentration) is one of the most preferred [7]. PULCON is an qNMR method which correlates the absolute intensities of two different spectra. Concentration measurements with PULCON use the principle of reciprocity which indicates that the lengths of a 90° or 360° pulse are inversely proportional to the NMR signal intensity [7]. PULCON based qNMR method has been evaluated and demonstrated by several studies on various chemical pharmaceutical materials (CPMs) [8 - 14]. One of the analytical objects that ES qNMR usually targets is certified reference materials (CRMs) for instrumental analysis such as HPLC with UV, fluorescence and mass spectrometric detection. For complex, difficult-to-synthesize and precious CRMs, PULCON based ES qNMR method is the appropriate choice and has been successfully applied in many fields of analytical chemistry [15 - 20].

Although PULCON based ES qNMR method has the merit, it was not easy to approach to this method in the laboratory because it requires additional installation of hardware. The Electronic REference To access In vivo Concentrations (ERETIC) is PULCON based tool, which does not require any additional hardware and ERETIC-2 tool is available on all major Bruker's NMR spectrometers [21]. The ERETIC tool provides an external reference signal that is generated electronically during the acquisition and can be placed anywhere in the NMR spectrum. After calibration, the concentration of a given molecule in the NMR sample can be determined by comparing the intensity of one of its signal to the intensity of the reference signal [22].

Compared to quantitative determination using IS qNMR method, the literature on ES qNMR is not as much. In particular, to our knowledge, there have been no reports on qNMR method application in Viet Nam. Therefore, the aim of this work was to develop and validate the ES qNMR method based on PULCON principle using ERETIC-2 tool to determine concentration and purity of organic compounds through the testing of two certified reference materials (CRMs) and three highly interested chemical pharmaceutical materials (CPM) extracted in Viet Nam, thereby establishing procedure for PULCON based ES qNMR method and assessing the applicability of this potential and promising analysis method for the analysis of Vietnamese medicinal herbs.

2. EXPERIMENTAL

2.1. RS, CRMs, CPMs and deuterated solvent

Six compounds, including a reference standard (RS), two CRMs and three CPMs have been prepared for PULCON based ES qNMR (Table 1). RS and CRMs of 98 % or 99 % purity are commercial products, originating from Energy Chem and Damas Beta (Shanghai, China). These compounds were often used as suitable RS for targeted quantitative analysis by means of HPLC, GC or capillary electrophoresis. The CPMs are natural products, extracted and provided by our colleagues at the Institute of Chemistry (ICH), Vietnam Academy of Science and Technology (VAST) with a purity of over 60 %, according to the suppliers.

NMR solvent was deuterated DMSO, 98 % D atom (Deutero GmbH, Kastellaun, Germany). All solution samples (RS and CRMs) were prepared by micropipettes (Nichipet EX-II) in volumetric flasks, providing complete dissolution in the NMR deuterated solvent, while all solid samples (CPMs) were prepared and weighed randomly using two analytical balances (Shimadzu AUW120D and Ohaus PA413) to minimize any potential weighing error.

Table 1. RS, CRM and CPM samples.

Sample	Compound	Chemical formula	MW (g/mol)	Purity (%)	Sample amount	Trade mark
RS	2-flourobezandehyde	C_7H_5FO	124.12	99	0.01 ml	Energy Chem
CRM-1	2-acetylthiophene	C_6H_6OS	126.18	99	0.01 ml	Energy Chem
CRM-2	4-methylmorpholine	$C_5H_{11}NO$	101.15	98	0.01 ml	Damas Beta
CPM-1	Curcumin	$C_{21}H_{20}O_6$	368.38	> 80	2 mg	-
CPM-2	Quercetin	$C_{15}H_{10}O_7$	302.326	60 -70	2 mg	-
CPM-3	Rutin	$C_{27}H_{30}O_{16}$	610.52	> 70	2 mg	-

2.2. ^{1}H qNMR experiments

RS, CRM and CPM samples were dissolved in deuterated solvents, well-mixed and free from bubbles by ultrasound. For all qNMR experiments, 0.6 mL of each solution was transferred to a dry precision glass 5 × 178 mm NMR tube (Deutero GmbH, Kastellaun, Germany) then covered with a polyethylene cap and wrapped with parafilm for making a tight seal.

^{1}H qHNMR sectra were performed at 298 K on an AVANCE III HD system (Bruker, Germany) operating at a frequency of 500.13 MHz, equipped with a Z-gradient 5 mm multinuclear observe probe (BBFO), using Bruker's parameter set of CMCQ_PROTON, which was set exclusively by Bruker for ERETIC-2 qNMR method. All spectra were referenced to TMS, acquired without spinning with a 16 scans of 64 K complex data points. All acquired parameters were kept constant for all experiments, only T1 relaxation times were determined for each sample using the inversion recovery pulse sequence. The relaxation delay for qNMR experiments was set to about 5 times the longest T1 relaxation time in the samples to ensure complete relaxation [22]. The data were automatically acquired under the control of ICON-NMR and sample track (Bruker, Germany), requiring 12 mins per spectrum. ^{1}H qNMR experiments were based on triplicate measurements, taking the average values of spectral integration, which is evaluated by relative standard deviation (RSD). The RS for qNMR analysis will be selected based on the criteria of purity, inertness, and stability after analysis of preliminary measured spectra.

2.3. Purity analysis

TopSpin 3.5 Bruker licensed software was used for ^{1}H NMR acquisition, the updated TopSpin 4.0.8, free version for academic users, was used for ERETIC-2 processing and

calculation regarding the software license issue. The free induction decay (FID) was multiplied with a 0.3 Hz exponential line-broadening factor prior to Fourier transformation. All NMR spectra were automatically phased, baseline-corrected and manually integrated.

The accuracy of the analytical results is compared with the commercial parameters of the product and the results of IS qNMR analysis. The precision of the analytical results is assessed based on RSD, calculated from three selected resonances on the same ^{1}H NMR spectrum.

3. RESULTS AND DISCUSSION

3.1. Reference standard for PULCON based qNMR method

Typical ^{1}H NMR spectra of 6 compounds (RS, CRM-1, CRM-2, CPM-1, CPM-2 and CPM-3) for ES qNMR experiment and mixed sample (RS + CPM-1, mole ratio of CPM-1: RS = 4:1) for IS qNMR experiment were showed in Figure 1. Based on the preliminary analysis of ^{1}H NMR spectra of commercial samples, 2-flourobezandehyde compound was selected as RS for both IS qNMR and ES qNMR methods due to its high purity (99 %), high signal-to-noise (S/N) ratio and the presence of single resonance (singlet, s) at down field spectral region, and separated from the resonances of all analytes.

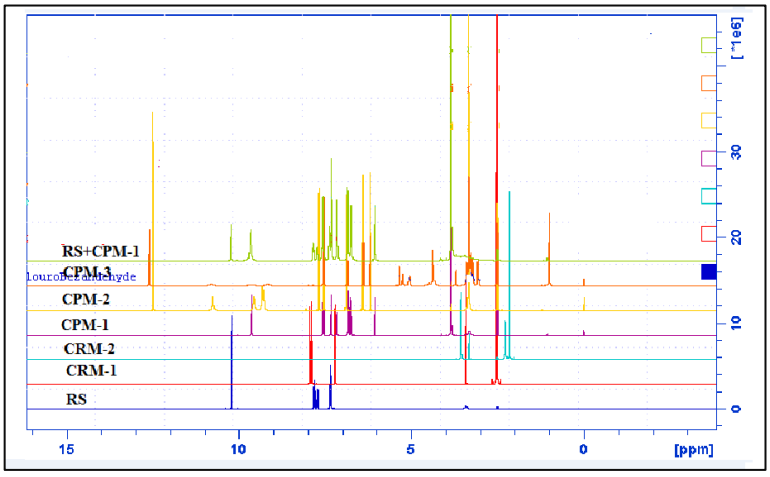

Figure 1. Typical ^{1}H qNMR spectra of RS, CRMs, CPMs and RS+CPM-1 samples.

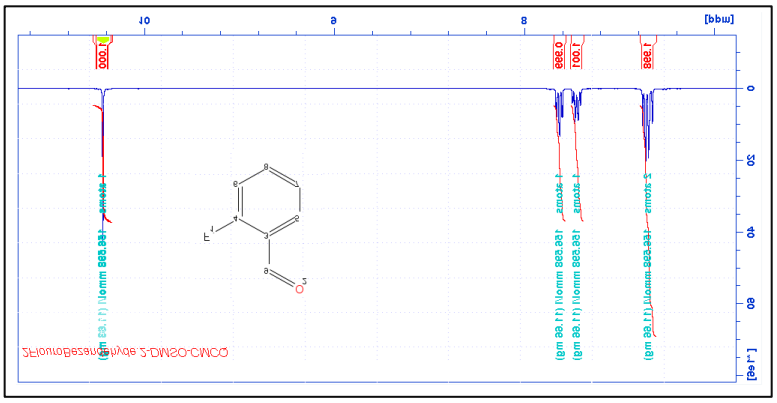

Figure 2. ^{1}H qNMR spectrum of 2-flourobezandehyde.

The ^{1}H NMR spectrum of 2-flourobezandehyde was shown in Figure 2. The spectrum consists of 5 resonances of 5 protons, although the NMR spectrum was measured in DMSO, two resonances at low field were overlapped. The resonances were multiplets, except for the resonance at down field (10.218 ppm). Two procedures of reference integration were caried out on ERETIC-2 module. In the first procedure, called the 1H procedure, one proton resonance (1H; s; 10.218 ppm) was chosen for integration, while in the second procedure, called the 5H procedure, the reference integration was the average of all 5 protons.

Three input parameters of ERETIC-2 for RS (2-Flourobezandehyde) were molecular weight (MW = 124.12 g/mol), sample volume (V = 0.6 ml) and mole concentration. The last was calculated according to the formula:

$$C = \frac{V \times D \times P}{MW \times V\ tube} = \frac{0.01\ (ml) \times 1.178(\frac{g}{ml}) \times 99(\%)}{124.12(\frac{g}{M}) \times 0.6(ml)} = 156.598 \times 10^{-6}(\frac{M}{mL}) = 156.598(\frac{mM}{L})$$

where C = Mole concentration; V = Volume of solution; D = Density; P = Purity; and V tube = Volume of solution in the NMR tube.

3.2. PULCON based qNMR analysis of CRMs and CPMs

Typical ERETIC-2 analyzed result by 5H procedure for CRM-1 sample was shown in Figure 3. The detailed result by both 1H and 5H procedures for this sample was shown in Table 2. Mole concentration was calculated by ERETIC-2 for three selected resonances (7.955 ppm, 1H, dd; 7.898 ppm, 1H, dd and 7.218 ppm, 1H, dd). The CH_3 resonance at 2.532 ppm could not be integrated because the peak was overlapped with DMSO solvent. The sample amount used for analysis was M_o= 0.01 (ml) × 1.168 (mg/ml) = 11.68 (mg). The purity was calculated from ERETIC-2 analyzed result ($P = 100 \times M_{ERETIC\text{-}2}/M_o$).

A deviation of about ± 0,68 % for purity, calculated using a 5H procedure is acceptable for qNMR analysis [16]. This deviation was related to the used of mean integral of all four observed signals in the spectrum as the reference data and it mainly depends on the integration subjectivity and spectral quality. Although ERETIC-2 tool provided an option of using the mean integral procedure (5H procedure), however, in practice this solution is of little use [22]. Thus, in the next section, the ERETIC-2 calculation will be performed based on the integral data of the singlet at 10.218 ppm (1H procedure), and there will be no deviation due to average integration.

Table 2. ERETIC-2 qNMR data of CRM-1 sample.

Procedure	NMR Peak (ppm)	H	$C_{ERETIC\text{-}2}$ (mM/L)	$M_{ERETIC\text{-}2}$ (mg)	Purity (%)
5H	7.955 (dd)	1	150.721 ± 1.051	11.411 ± 0.080	97.70 ± 0.68
	7.898 (dd)	1	150.882 ± 1.054	11.465 ± 0.080	97.80 ± 0.68
	7.218 (dd)	1	151.384 ± 1.053	11.506 ± 0.080	98.12 ± 0.68
1H	7.9530 (dd)	1	151.616	11.479	98.28
	7.8959 (dd)	1	151.778	11.491	98.38
	7.2202 (dd)	1	152.283	11.529	98.71

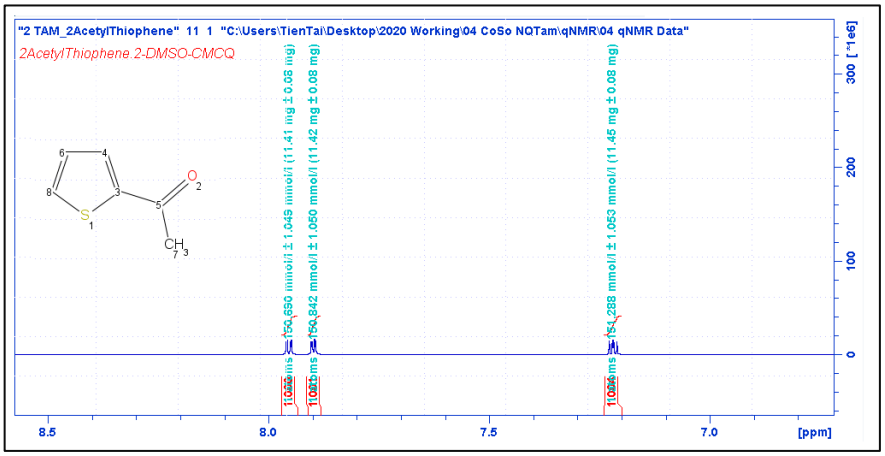

Figure 3. Typical analyzed ERETIC-2 ^{1}H qNMR spectrum of CRM-2 sample.

Table 3. ERETIC-2 qNMR data and reference purity data of CRMs and CPMs samples.

Sample	NMR Peak (ppm)	Purity (%)			
		By PULCON based ES qNMR		By IS qNMR	By Manufacturer
CRM-1	7.9530 (dd)	98.28	98,45	98,72	99
	7.8959 (dd)	98.38	(0.23 % RSD)	(0.65 % RSD)	
	7.2202 (dd)	98.71			
CRM-2	3,5613 (t)	97.27	97.26	97.46	98
	2.2920 (t)	96.89	(0.38 % RSD)	(0.48 % RSD)	
	2.1580 (s)	97.64			
CPM-1	9.6459 (s)	85.18	84.86	83.93	> 80
	7.3382 (dd)	82.78	(2.46 % RSD)	(2.20 % RSD)	
	3.8533 (s)	86.93			
CPM-2	7.7071 (d)	56.11	56.86	59.10	60 -70
	6.4337 (d)	57.61	(1.50 % RSD)	(1.34 % RSD)	
	6.2011 (d)	57.57			
CPM-3	6.8682 (d)	75.25	78.17	76.40	> 70
	6.4410 (d)	78.23	(3.73 % RSD)	(2.65 % RSD)	
	6.2015 (d)	81.08			

Determination of purity for the remain CRM and CPM samples with the ERETIC-2 tool was carried out in the same way as for the CRM-1 sample. The results were summarized in Table 3. The purity of the samples has also been determined by IS qNMR technique for comparison and evaluation. 2-fluorobenzaldehyde was used as RS for both IS qNMR and ES qNMR methods. ^{1}H NMR spectra of RS and CPM-1 samples by ES qNMR experiment and spectrum of RS + CPR-1 sample by IS qNMR experiment were shown in Figure 4.

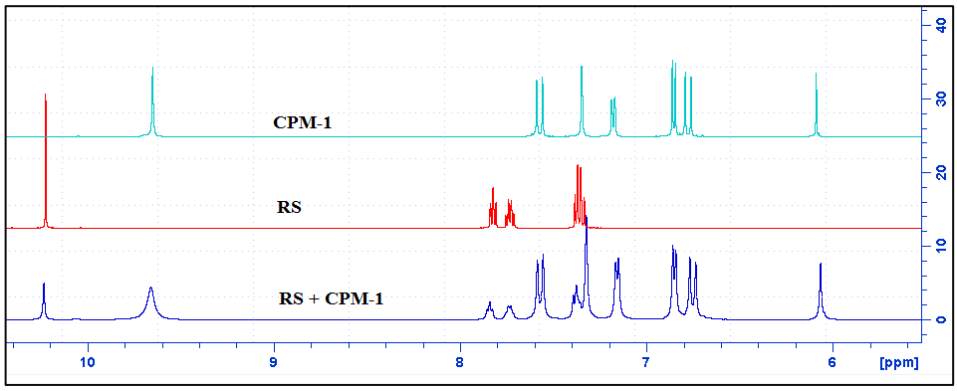

Figure 4. ^{1}H NMR spectra of RS, CPM-1 and RS+CPM-1 samples.

For two commercial CRM samples, small molecule with high purity and simple structure, PULCON based ES qNMR analysis results (Table 3) showed that the errors were in the range of 0.2 - 0.3 % and 0.5 - 0.8 % when compared with the IS qNMR result and manufacturer's data, respectively. Moreover, the RSD of PULCON based ES qNMR results was relatively low (0.23 - 0.38 %), which equals to the RSD of IS qNMR method. For CPM samples, large molecule with complex structure and complex NMR spectra, the errors for all 3 samples were significantly higher than that of CRMs, the deviation was in the range of 1.5 - 3.8 %, deviation from IS qNMR data was 1.34 - 2.65 %, the purity determined by both ES- and IS qNMR methods was within the data expected from sample suppliers.

The factors contributing to the accuracy and precision of ES qNMR are generally divided into three main groups [16]: experimental factors, RS and ERRETIC. Related to the first factor, all ^{1}H NMR measurements were accurately tuned and matched at the observing frequency, NMR experiment temperature was kept for constant at 298 K, adequate relaxation delays (about $5T_1$) were used, the RSD for the triplicate experiments was small (< 2 %). The contribution from the RS factor is mainly related to the purity of 2-flourobezandehyde (99 %) and the procedure of RS integration. The choice of ^{1}H integration procedure based on singlet at 10.218 ppm minimized the effect of RS integral. Therefore, the major contributions to the accuracy and precision arised from integration subjectivity, which is highly sample-dependent. For CPMs that have more complex spectra and may contain residual protonated impurities, the results were influenced by the choice of peaks for integration and baseline correction, in this case, the result can be obtained by the average of two or more integrated peaks on the spectrum.

In general, for qNMR analysis, an accuracy within < 1 % is acceptable for samples with high purity (> 90 %), a lower accuracy is only meaningful for the rapid measurement or screening of natural compounds. Precision is typically less than 2 % for high purity or synthetic samples and even up to 6 % for natural substances [14, 17]. The obtained accuracy and precision from our work (Table 3) suggested that the ERETIC-2 ES qNMR method could be used for quantitative analysis of both CRM and CPM samples.

4. CONCLUSIONS

An experimental procedure for PULCON based ES qNMR method was initially established in a NMR Lab in Viet Nam. The results using this method for determination of the purity of CRM and CPM samples indicated that the accuracy and precision of the method are acceptable, and ERETIC-2 ^{1}H qNMR can be considered as a practical alternative tool with competitive accuracy, precision and other advantages. Therefore, PULCON based ES qNMR method can be applied for routine quantitative analysis of synthetic or natural compounds, especially, when the availability and the costs of reference standard are problematic. In addition, extensive application of this analytical tool for analysis of mixed samples and metabolomics is also a promising and attractive orientation.

Acknowledgments. This research is funded by the Institute of Chemistry, Vietnam Academy of Science and Technology under a grant (2020 basic project). The authors are grateful to Dr. Vu The Hien and MSc. Vo Ngoc Binh at ICH, VAST for the supply of RS, CRM and CPR samples.

CRediT authorship contribution statement. Nguyen Quang Tam: Analysis and Supervision, Dang Vu Luong: Experiments, Do Thi Thanh Xuan: Experiments, Thanh Thi Thu Thuy: Methodology, Nguyen Tien Tai: Analysis and Methodology.

Declaration of competing interest. There is no conflict of interest.

REFERENCES

1. Santosh Kumar Bharti, Raja Roy - Quantitative 1H NMR spectroscopy, TrAc Trends in Analytical Chemistry **35 (**2012) 5-26.
2. Holzgrabe U., Deubner R., Schollmayar C., Waibel B. - Quantitative NMR spectroscopy - Application in drug analysis, J. Pharm. Biomed. Anal. **38** (2005) 806-812.
3. Rizzo V., Pinciroli V. - Quantitative NMR in synthetic and combinatorial chemistry, J. Pharm. Biomed. Anal. **38** (2005) 851-857.
4. Guido F. Pauli, Birgit U. Jaki and David C. Lankin - Quantitative 1H NMR: Development and Potential of a Method for Natural, Products Analysis§ J. Nat. Prod. **68** (2005) 133-149.
5. Guido F. Pauli et al. - Importance of Purity Evaluation and the Potential of Quantitative 1H NMR as a Purity Assay, J. Med. Chem. **57** (2014) 9220-9231.
6. Gregory K. Webster and Shailendra Kumar - Expanding the Analytical Toolbox: Pharmaceutical Application of Quantitative NMR, Anal. Chem. **86** (2014) 11474−11480.
7. Wider G. and Dreier L. - Measuring Protein Concentrations by NMR Spectroscopy; J. Am. Chem. Soc. **128** (2006) 2571-2576.
8. Hong R. S., Hwang K. H., Kim S., Cho H. E., Lee H. J., Hong J. T., Moon D. C. - Survey of ERETIC2 NMR for quantification, J. Kor. Magn. Reson. **17** (2013) 98-104.
9. Marie Emilie Wekre, Karoline Kåsin, Jarl Underhaug, Bjarte Holmelid and Monica Jordheim - Quantification of Polyphenols in Seaweeds: A Case Study of Ulva intestinalis, Antioxidants **8** (2019) 612-627.
10. Nerantzaki A. A., Tsiafoulis C. G., Charisiadis P., Kontogianni V. G., Gerothanassis I. P. - Novel determination of the total phenolic content in crude plant extracts by the use of 1H NMR of the–OH spectral region, Anal. Chim. Acta **688** (2011) 54-60.

11. Chauthe S. K., . Sharma R. J, Aqil F., Gupta R. C., Singh I. P. - Quantitative NMR: An applicable method for quantitative analysis of medicinal plant extracts and herbal products, Phytochem. Anal. **23** (2012) 689-696.
12. Li-Ying Hsieh et al. - A feasible and practical 1H NMR analytical method for the quality control and quantification of bioactive principles in Lycii Fructus, Journal of food and drug analysis **26** (2018) 1105-1112.
13. Watanabe R, Sugai C, Yamazaki T, Matsushima R, Uchida H, Matsumiya M, et al. - Quantitative nuclear magnetic resonance spectroscopy based on PULCON methodology: application to quantification of invaluable marine toxin, okadaic acid, Toxins **8** (2016) 294-303.
14. Salem A. A., Mossa H. A. - Method validation and determinations of levofloxacin, metronidazole and sulfamethoxazole in an aqueous pharmaceutical, urine and blood plasma samples using quantitative nuclear magnetic resonancespectrometry, Talanta **88** (2012) 104–114.
15. Weber M., Hellriegel C., Rueck A., Sauermoser R., J. Wuethrich- Using highperformance quantitative NMR (HP-qNMR) for certifying traceable and highly accurate purity values of organic reference materials with uncertainties <0.1 %, Accred. Qual. Assur. **18** (2013) 91-98.
16. M. Weber, C. Hellriegel, A. Rueck, J. Wuethrich, P. Jenks - Using high-performance 1H NMR (HP-qNMR) for the certification of organic reference materials under accreditation guidelines - Describing the overall process with focus on homogeneity and stability assessment, JPBA **93** (2014) 102-110.
17. Yulia B. Monakhova, Matthias Kohl-Himmelseher, Thomas Kuballa, Dirk W. Lachenmeier - Determination of the purity of pharmaceutical reference materials by ^{1}H NMR using the standardless PULCON methodology, Journal of Pharmaceutical and Biomedical Analysis **100** (2014) 381-386.
18. Monakhova Y.B., Kohl-Himmelseher M., Kuballa T., Lachenmeier D.W. - Determination of the purity of pharmaceutical reference materials by ^{1}H-NMR using the standardless PULCON methodology. J. Pharm. Biomed. Anal. **100** (2014) 381-386.
19. Ian W. Burton, Michael A. Quilliam and John A. Walter- Quantitative ^{1}H NMR with External Standards: Use in Preparation of Calibration Solutions for Algal Toxins and Other Natural Products, Anal. Chem. **77** (2005) 3123-3131.
20. Oliver Frank, Johanna Karoline Kreissl, Andreas Daschner, and Thomas Hofmann - Accurate Determination of Reference Materials and Natural Isolates by Means of Quantitative ^{1}H NMR Spectroscopy, J. Agric. Food Chem. **62** (2014) 2506-2515.
21. TopSpin ERETIC-2, User Manual, Version 001, Bruker Corporation, (2016).
22. Akoka S., Barantin L., Trierweiler M. - Concentration measurement by proton NMR using the ERETIC method, Anal. Chem. **71** (1999) 2554–2557.