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A Simple and Feasible Method for the Quantification of Metabolites in the Human Follicular Fluid Using ^{1}H HR-MAS NMR Spectroscopy

Akın MUMCU[1,2*]

[1]Laboratory of NMR, Scientific and Technological Research Centre, Inonu University, Malatya, Turkey

[2]Reproductive Sciences & Advanced Bioinformatics Application & Research Centre, Inonu University, Malatya, Turkey

Abstract: This study presents a reliable method for the quantification of metabolite concentrations in follicular fluid with the high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy and the ERETIC2 (Electronic REference To access In vivo Concentrations) based on PULCON (pulse length based concentration determination) principle. The positive effect of the HR-MAS probe technology on spectral quality and its ability to perform analyses with very low sample amounts were the most important factors of proposing this method. In evaluating the performance of the proposed method, standard creatine solutions in different concentrations containing DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt) as an internal reference standard were analyzed using different pulse programs (cpmgpr1d and zg30). The results obtained with the ERETIC2 were compared with the classical internal standard NMR quantification method (DSS method). The relative standard deviation (RSD) values for ERETIC2 were in the range of 0.3% - 5.7% and recovery values were calculated as minimum 90.3%, while RSD values for DSS method were in the range of 0.1% - 3.1% and recovery values were minimum 97.0%. Besides, it was observed that the metabolite concentration values calculated using the ERETIC2 procedure of follicular fluid samples obtained from the women with endometriosis and healthy controls were compatible with the values those obtained using different methodologies. The obtained results showed that the proposed quantification method based on the HR-MAS spectroscopy can easily be used in biological fluids and therefore it can be utilized as a good alternative to the internal standard method considering its accuracy and precision.

Keywords: HR-MAS spectroscopy, quantification, PULCON, ERETIC2, metabolite concentration

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***Corresponding author. E-mail:** akinmumcu@gmail.com

INTRODUCTION

High-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy is a versatile analytical technique and it was developed to obtain high-quality NMR spectra in studies on semi-solid and heterogeneous systems (1). Nuclear spins in semisolid samples are subject to some interactions resulting in dominant spectral broadening (2). This spectral broadening results from chemical shift anisotropy, magnetic susceptibility, and dipolar-quadrupolar interactions caused by sample heterogeneity

(3). These line broadening effects are reduced by the fast spinning of the sample in the direction of a static magnetic field at the angle of 54.7° in the HR-MAS technique and a high-resolution NMR spectrum is obtained (4,5). With these features, HR-MAS spectroscopy is an analytical tool of increasing importance in the characterization of metabolic structure in heterogeneous samples such as intact tissues and cells (6). Besides, since almost all biological fluids except urine do not fall into the classical definition of aqueous solution due to their protein or macromolecule contents, the broadened NMR signals in these fluids can be reduced by using the HR-MAS technique (7). Metabolic characterization, also called metabolomics, is the systematic identification and quantification of metabolites to obtain important biochemical information from various biological samples or a given organism (8). The ability to measure metabolite concentrations, especially in biological samples, is crucial for examining the pathophysiology and course of a disease, monitoring its response to treatment, and classifying disease states (9). Quantification of metabolite concentrations not only allows us to identify abnormal metabolism but also helps to compare results from different subjects and patient groups (9). In this context, quantitative NMR has been used for many years by different disciplines in determining biochemical pathways of plants and animal species as well as the identification of biomarkers for the diagnosis of diseases (10). There are two main types of quantification with NMR: relative and absolute quantification (11). Although relative quantitation is used in many metabolomics studies, it has a major disadvantage. In this method, concentrations of related metabolites simultaneously change in some disease states. Therefore, absolute quantitation is preferred for clinical diagnosis and determination of metabolite concentrations in physiological studies (12). There are two main NMR methods, depending on whether an internal or external reference standard is used for absolute quantification (11). The internal reference method is simple and easy to use (13). However, it is not always applied in the sample solution, as it is difficult to create the correct combination of the solvent, analyte, and internal standard, without any chemical interaction between them (11). Addition of internal NMR reference chemicals such as DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt) or TSP (trimethylsilyl propionate), which are mostly used in biological applications, carries the risk of chemical interaction of these substances with biological macromolecules in the sample. Also, quantification becomes difficult due to overlap signal problems (14). The use of the external reference method can avoid these disadvantages of the internal method (11). In the external reference method, the reference compound and sample are prepared as separate solutions and NMR signals are measured simultaneously in two separate NMR tube systems. This method prevents possible interactions between the reference substance and biological macromolecules but requires additional sample preparation time (13). Another disadvantage, since the external reference method depends on concentrations, an additional source of error may occur that can be caused by volume measurements of the solvents used in the preparation of calibration and sample solutions (11). An alternative approach to the external reference method was first introduced by Barantin et al. as a new method in vivo quantification (15). This method, called the ERETIC (Electronic reference to access in vivo concentrations) method, uses an artificial NMR signal produced by a small loop coil that matches the RF coil as a reference signal (12). Since the ERETIC method uses a synthesized RF pulse during the acquisition period when generating a reference signal, (16) there is no need to add any internal reference material to the sample (17). However, the need for some modifications in the spectrometer setup, the requirement additional specialized hardware and the necessity of the ERETIC signal to remain stable regularly are the drawbacks of this method (14). Also, several important quantification methods are available, such as PULCON (PULse-length-based CONcentration determination) and ERETIC2 (Bruker Topspin) (13). PULCON is an internal standard method (18) that relates the absolute intensities of two different spectra and uses the reciprocity principle, which indicates that the 90° or 360° pulse lengths is inversely proportional to the NMR signal intensity (14). This method provides the necessary compensation factors for loses in coil sensitivity originating from dielectric properties in different samples and does not require specialized electronic devices (19). While the development of the PULCON method brought a noticeable convenience to the applicability of quantitative NMR, it also established an infrastructure for the ERETIC2 method which is based on the

PULCON principle. ERETIC2 is the name of a software program developed by Bruker that uses the PULCON principle to determine the absolute concentration of a molecule (20). ERETIC2 does not require specialized NMR tube systems and any additional devices to generate an electronic reference signal. Also, it prevents any interaction between the analyte and the reference compound as well as eliminating the problems caused by peak overlaps (13). With these features, the ERETIC2 is a good alternative to the classical internal standard method due to the convenience provided to researchers in the absolute quantification of the unknown substance (18). This study aims to evaluate the accuracy and precision of the ERETIC2 in the quantification of the metabolite composition of follicular fluid samples obtained from participants with endometriosis and healthy using HR-MAS spectroscopy. Human follicular fluid was chosen because it contains important metabolites that affect oocyte quality, fertilization, and embryonic development and provides important information about folliculogenesis (21,22). Moreover, it is thought that the changes in the metabolic profile of the follicular fluid (FF), which constitutes the microenvironment of oocyte, plays an important role in determining oocytic quality and embryonic quality, and subsequent embryonic development and fertilization (23,24). In recent years, the recognition of the relationship between follicular fluid (FF) and oocyte quality and the ability to obtain follicular fluid easily with oocyte during standard in-vitro fertilization procedures have attracted the interest of omics-based technologies and different disciplines (25,26). To the best of our knowledge, this is the first study in literature that utilizes ERETIC2 and HR-MAS technique for quantification of the metabolite content of follicular fluid. To achieve this, firstly the accuracy of ERETIC2 was compared with the classical internal standard method. For this purpose, different pulse sequences and different concentrations of standard solutions containing DSS as an internal reference standard were used. The ERETIC2 was then applied to follicular fluid samples for metabolite quantification.

EXPERIMENTAL SECTION

Preparation of standard solutions

Firstly, a stock solution containing 10 mM creatine and DSS was prepared using 10 mM phosphate-buffered saline (PBS, pH 7.4) prepared in D_2O (deuterium oxide). Standard solutions of 10 mM, 5 mM, 3 mM, and 1 mM to be used in the calibration experiments were prepared with dilution weight by volume from 10 mM stock solution. The phosphate buffer salt prepared in D_2O was used in the dilution process. Chemicals used in this study, such as the creatine monohydrate (%99), D_2O (%99.9 D atom, 1.11 g/mL), KH_2PO_4 (%98) and Na_2HPO_4 (%99) were obtained from Merck, while the DSS (%97), KCl (%99) and NaCl were obtained from Sigma-Aldrich. Sartorius CP225D (readability 0.01 mg) was used for all weight measurements.

Sample preparation for HR-MAS spectroscopy

Follicular fluids used in HR-MAS analysis were obtained from the patients recruited in İnönü University Faculty of Medicine, Department of Obstetrics and Gynecology. This study was approved by the Human Research Ethics committee of Malatya (No: 2016/113), and participants was enrolled in the study after their written informed consent. The healthy control sample was selected from the patients having intracytoplasmic sperm (ICSI) treatment, while the sample with endometriosis was selected from the patients having in vitro fertilization (IVF) treatment. Thus, this study includes follicular fluid samples collected from a total of 10 patients. Five of the samples were obtained from healthy controls and 5 from patients with endometriosis. The diagnosis of endometrioma in patients with endometriosis was made using transvaginal ultrasonography. The healthy control group was selected only from patients with infertility caused by a strong male factor (severe azoospermia). Individuals participating in the study had good physical and mental health. The criteria for inclusion of individuals with normal pelvic anatomy as a result of ultrasonographic screening were age < 35, BMI (body mass index) < 30 kg/m^2 and FSH (follicle-stimulating hormone) < 10.0 IU/L. As exclusion criteria, age of > 35, BMI > 30 kg/m^2, FSH > 10.0 IU/L and chronic systemic diseases were chosen. Follicular fluid samples to be NMR analyzed were collected from approximately 20 mm sized follicles during oocytic recovery and added to individual sterile tubes. All samples were centrifuged for 10 min at 2900 rpm to remove possible cell impurities. After these procedures, samples were stored at -80 °C until NMR analysis (27).

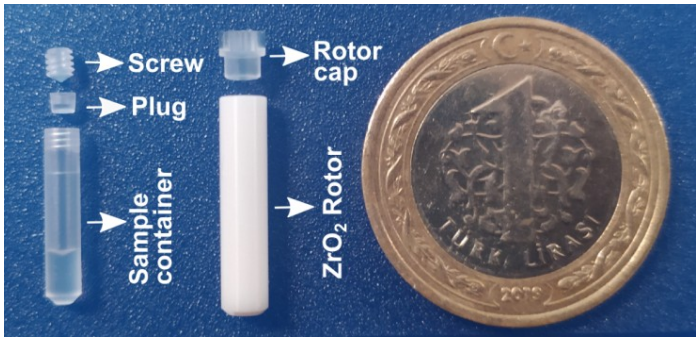

Figure 1: 4 mm diameter zirconium rotor and disposable HR-MAS insert consisting of three parts (screw, plug and container). The plug seals the sample inside the sample container. The plug restraining screw holds the plug in position. The assembled insert fits into a standard HR-MAS rotor.

HR-MAS analyses of both standard solutions and follicular fluids were performed in 30 µL volumes in sealed disposable inserts (Bruker) made from biologically inert KEL-F (poly (1-chloro-1,2,2-trifluoroethylene). Since creatine solutions of different concentrations are prepared in D_2O, samples of 30 µL volume taken from them are placed first in disposable inserts consisting of one sample container, plug, and screw, and then in a 4 mm diameter ZrO_2 MAS rotor suitable for these inserts (Figure 1). For follicular fluid samples, first 5 µL of D_2O and then 25 µL of FF were added into the disposable insert and after the rotor assembly was completed, samples were transferred to the HR-MAS probe for NMR analysis. A possible error during the assembly of the rotor may cause some spin problems during the analysis or loss of the lock signal as a result of sample leakage. To avoid this, the amount of sample in the rotor was double-checked by weighing before and after the analysis.

^{1}H HR-MAS analysis of samples

All one-dimensional (1D) ^{1}H NMR experiments were recorded using a 600 MHz Bruker Avance III HD NMR spectrometer equipped with a 4 mm diameter HR-MAS probe (^{1}H/^{13}C/^{15}N). All samples were spun at 4 kHz using a MAS speed controller. While the probe temperature was controlled using a Bruker cooling unit, instrumental temperature in all experiments was retained constant at 295 K. When the samples were placed in the magnet, HR-MAS probe was manually tuned and matched to reduce radio frequency (RF) reflection before each analysis. ^{1}H NMR spectra of each creatine solution samples were acquired using both standard zg30 pulse sequence (RD - 30° - FID) and Carr-Purcell-Meiboom-Gill (cpmgpr1d) pulse sequence (RD - 90° - {180° - τ - (τ - 180° - τ)$_L$ - τ - 180°} - FID), whereas ^{1}H NMR spectra of follicular fluids (FF) samples were acquired using only cpmgpr1d pulse sequence. During standard zg30 pulse experiments, 64K data points and a spectral width of 12019.2 Hz were applied. For each sample, FID (free induction decay) acquisitions were collected in 8 min using 2.73 sec acquisition time, 128 transients, and a relaxation delay (RD) of 1.0 sec. All CPMG ^{1}H NMR spectra were recorded with 32K data points and a spectral width of 7002.8 Hz. Where CPMG echo delay (τ) was 0.7 msec, relaxation delay (RD) was 4.0 sec, and the number of loops (L) was 128. The water signal was suppressed with a weak presaturation pulse (2.8E-5 W) on water peak frequency during the acquisition. FID acquisitions were collected in 27 min using 2.34 sec acquisition time and 256 transients. Bruker Topspin (ver. 3.2.7) was used for the processing of NMR data. Previous to Fourier transform, the exponential line broadening of 0.3 Hz was performed to FID. All spectra were manually phase-corrected and baseline corrected. The chemical shifts of creatine samples were calibrated according to the singlet peak at 0 ppm of the DSS reference. Spectral assignments of follicular fluid metabolites were determined from the published literature and from various resources (28,29).

Comparison experiments of the internal standard method (DSS method) and ERETIC2

The concentrations of creatine solutions in different concentrations (10 mM, 5 mM, 3 mM, and 1 mM) containing DSS as the internal

reference standard substance was calculated using both the internal standard method (DSS method) and the ERETIC2. First, Equation 1 was used to calculate the concentrations of creatine samples using the internal standard method.

$$[X_{Cr}] = n_{DSS} / n_{Cr} \times A_{Cr} / A_{DSS} \times [DSS] \quad \text{(Eq. 1)}$$

In this equation, n_DSS and n_Cr show the proton numbers of the DSS and creatine peaks, respectively. In concentration calculations, 9 proton singlet signals of DSS in the range of 0.02 to - 0.17 ppm and 3 proton methyl signal of creatinine in the range of 3.05 - 2.9 ppm were used. A_Cr and A_DSS show the calculated integral area of the creatinine methyl peaks and the DSS peak. [DSS] is the concentration of DSS in the sample. Secondly, the quantification module called ERETIC2 developed by Bruker was used in concentration calculations. ERETIC2 was carried out in two steps including calibration and quantification. In the calibration step, a 10 mM DSS solution prepared in D_2O solvent containing 10 mM phosphate-buffered saline was used as the ERETIC2 reference to generate the synthetic signal. NMR spectrum obtained from ERETIC2 reference sample was recorded using acquisitions parameters such as number of scan (ns) and receiver gain (rg) specified for this sample. The signals in the DSS spectrum are defined as ERETIC reference from the integration menu of the ERETIC2 module in the software program of TopSpin and the calibration process is completed. In the quantification step, NMR spectra from creatine solutions prepared at different concentrations were recorded using the same experimental parameters as the ERETIC2 reference sample except for receiver gain. The receiver gain and the 90° pulse length were determined individually for each sample. In addition, the same processing parameters were used in both steps (supporting information file Figure S1). The creatine concentrations in the sample were then determined by Equation 2 using the integration menu in the ERETIC2 module (18).

$$C_{Cr} = k \cdot C_{Ref} \frac{A_{Cr} \cdot T_{cr} \cdot \theta_{90}^{Cr} \cdot ns_{Ref}}{A_{Ref} \cdot T_{Ref} \cdot \theta_{90}^{Ref} \cdot ns_{Cr}} \quad \text{(Eq. 2)}$$

In the Equation 2 used by the ERETIC2, C_{Cr} and C_{Ref} are the concentrations of the creatine and reference samples respectively, T is the temperature at which analyses are performed, Θ_{90} 90° is the pulse length, *ns* is the number of scans used for experiments, and *k* is the correction factor accounts for the experimental differences such as the receiver gain or incomplete relaxation (18). To evaluate the accuracy of both methods, 1D ^{1}H NMR analyses of all samples were repeated five times (supporting information file Table S1 and Table S2), both in the same samples and in different samples prepared. While the analysis of the same samples was repeated, the samples were removed the magnet and placed back into the magnet each time.

Metabolite quantification in follicular fluid samples with ERETIC2

Similar to the quantification of creatine solutions, metabolite quantification with the ERETIC2 was also performed in two steps. In the calibration process, for the ERETIC2 reference, the previously obtained NMR spectrum of the 10 mM DSS solution was used. In the quantification step, the NMR spectra of the follicular fluid samples were used for metabolite quantification (supporting information file Figure S2). Also, 1D ^{1}H NMR analysis of each follicular fluid sample was repeated three times with different samples prepared.

RESULTS AND DISCUSSION

Spectral properties and advantages of HR-MAS

The metabolite peaks examined in NMR studies of biological fluids are overlapped by broad signals originated from proteins, lipids, and especially water, which is a predominant component of biological samples (4). Therefore, suppression of the water peak is very important to obtain interpretable NMR spectra (30). Figure 2 shows the NMR spectra recorded utilizing the identical pulse sequences of the same follicular fluid sample with the 4 mm diameter HR-MAS probe (Figure 2b) and the conventional 5 mm diameter liquid state NMR probe (Figure 2a). As seen in Figure 2, water suppression with the HRMAS probe is more successful than the water suppression with the conventional probe. In this way, with a better baseline obtained in the HR-MAS spectrum, the β-glucose (4.55 ppm) and α-glucose (5.13 ppm) signals located in areas close to the water signal at about 4.7 ppm are more clearly observed. This provides an advantage in the correct quantification of the relevant metabolite peaks. Water suppression is

improved by some experimental and hardware factors. In NMR spectroscopy, some gradient-based suppression techniques (WET, WATERGATE, or CPMG) which can be easily combined with most of the pulse sequences are used to suppress unwanted signals in the spectrum (31). Another important factor for successful water suppression is the homogeneity of the B_0 magnetic field (32). Better B_0 homogeneity can be achieved by reducing the sample volume and keeping the entire sample within the active volume of the probe (33). However, in conventional 5 mm diameter NMR probes, the receiver coil is shorter than the sample length in the NMR tube, and a solution volume of approximately 600 µL is required to provide the appropriate spectral resolution in these probes. This causes unsuppressed water in the sample to disperse inside or outside the active volume of the probe.

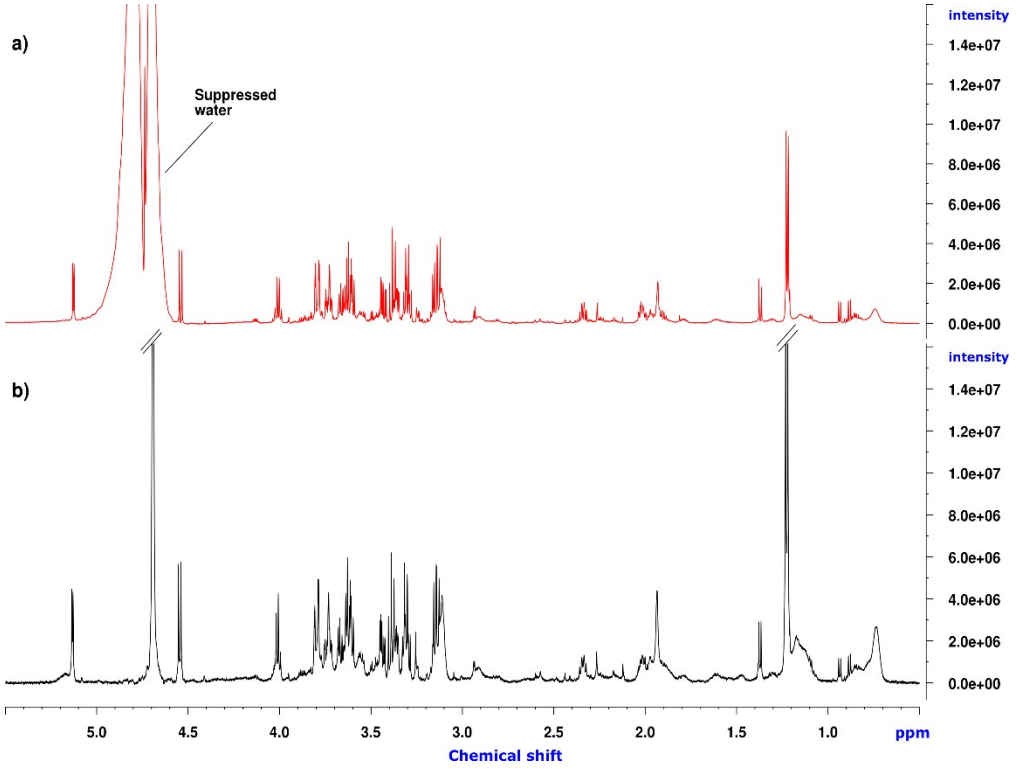

Figure 2: Effects of two different probes on solvent suppression using the same follicular fluid sample. 1D ^{1}H-NMR spectra acquired at 600 MHz using a) a 5 mm conventional liquid state probe and b) a 4 mm HR-MAS probe. While the watt value was retained between probes for presaturation, different values were used for pulse length and power level for pulse in both probes due to probe design differences. In addition, depending on the differences in the sample requirements of the probes, 400 µL of sample were used for the conventional liquid state probe, while only 25 µL of sample were used for the HR-MAS probe. The difference in metabolite signal intensities in the same follicular fluid sample can be easily seen.

In HR-MAS technology, which allows working with very low sample quantities (≤ 40 µL), the receiver coil of the HR-MAS probe head is slightly larger than the sample chamber of the probe, so the entire sample is placed in the coil active volume of the probe (34). This results in better B_0 homogeneity and better water suppression. Also, when Figure 2 is examined, it is seen that the noise level in the spectrum obtained with the conventional 5 mm diameter probe is slightly better than the HR-MAS probe. However, the intensity of the peaks in the spectrum obtained with the HR-MAS probe are higher. The signal-to-noise (S/N) ratio values of these two spectra in a spectral noise region of approximately 1000 Hz were calculated as 15.9 (Figure 2a) and 12.6 (Figure 2b), respectively. Although this depends on the sensitivity

characteristics of the probes studied (90° pulse in μs, power level in W, etc.), the detection limit of the HR-MAS technology is slightly better when compared to the conventional 5 mm diameter liquid state NMR probe under the most optimized conditions for the studied sample. It is possible to say that this situation is an advantage for samples with limited quantity, especially considering that a sample amount of approximately 25 μL is used in the HR-MAS technique. However, it should be noted that sample recovery from the HR-MAS rotor is more difficult compared to other small diameter NMR tubes.

Performance of NMR quantification methods

Quantification results of creatine concentrations in standard creatine solutions of different concentrations determined using DSS method and ERETIC2 are presented in Table 1. In both methods, creatine concentrations were calculated by means of methyl peaks using two different pulse sequences (cpmgpr1d and zg30). Also, the standard deviation (± SD), % recovery, and % relative standard deviation (RSD) values are provided for all results from the same sample and differently prepared samples at each concentration. Recovery was calculated with ({measured value / actual value} × 100), and the relative standard deviation was calculated with ({standard deviation / mean value} × 100). In Table 1, when 5 repeated measurement results of the same samples were examined in both methods, the lowest recovery value was determined as 97.0% in the DSS method for the cpmgpr1d pulse sequence, while the lowest recovery value was determined as 108.0% for the zg30 pulse sequence. In ERETIC2, a 90.3% recovery value was calculated for cpmgpr1d pulse sequence, and the lowest recovery value for zg30 pulse sequence was determined as 102.0%. Likewise, when the results obtained with the DSS method and the ERETIC2 for 5 differently prepared samples were compared, the lowest recovery value for the cpmgpr1d pulse sequence in the DSS method was 98.3%, while this value was determined as 108.0% with the zg30 pulse. In the ERETIC2, the lowest recovery values for cpmgpr1d and zg30 pulse sequences were calculated as 94.3% and 102.0%, respectively. Besides, when the values for the same samples provided in Table 1 are examined, RSD values of 1.2% (for the cpmgpr1d) and 2.4% (for the zg30) or lower were achieved in the DSS method. While in the ERETIC2, RSD values of 2.2% (for the cpmgpr1d) and 1.7% (for the zg30) or lower were obtained. Considering the results obtained from differently prepared samples, the DSS method provided 2.0% (for the cpmgpr1d) and 3.1% (for the zg30) or lower RSD. On the other hand, the ERETIC2 provided RSD values of 5.2% (for the cpmgpr1d) and 5.7% (for the zg30) or lower for these samples.

Table1: Quantification results determined using the DSS method and ERETIC2 from standard creatine solutions (C.S) in different concentrations (mM).

Method	Sample type	Pulse program	C.S (mM)	Concentrations (mM)		
				Mean (±SD)	Recovery (%)	RSD (%)
DSS method	from the same sample	cpmgpr1d	10	9.79 (± 0.02)	97.9	0.2
			5	4.99 (± 0.06)	99.8	1.2
			3	2.91 (± 0.02)	97.0	0.7
			1	0.98 (± 0.01)	98.0	1.0
		zg30	10	10.80 (± 0.01)	108	0.1
			5	5.44 (± 0.06)	108.8	1.1
			3	3.42 (± 0.02)	114.0	0.6
			1	1.27 (± 0.03)	127.0	2.4
	from the different prepared samples	cpmgpr1d	10	9.85 (± 0.13)	98.5	1.3
			5	4.92 (± 0.07)	98.4	1.4
			3	2.95 (± 0.03)	98.3	1.0
			1	1.01 (± 0.02)	101.0	2.0
		zg30	10	10.80 (± 0.01)	108	0.1
			5	5.44 (± 0.04)	108.8	0.7
			3	3.44 (± 0.03)	114.6	0.9
			1	1.28 (± 0.04)	128.0	3.1

ERETIC2	from the same sample	cpmgpr1d	10	9.51 (± 0.04)	95.1	0.4
			5	4.95 (± 0.11)	99.0	2.2
			3	2.71 (± 0.04)	90.3	1.5
			1	0.93 (± 0.02)	93.0	2.1
		zg30	10	10.20 (± 0.07)	102	0.7
			5	5.28 (± 0.06)	105.6	1.1
			3	3.12 (± 0.01)	104.0	0.3
			1	1.18 (± 0.02)	118.0	1.7
	from the different prepared samples	cpmgpr1d	10	9.43 (± 0.16)	94.3	1.7
			5	4.93 (± 0.05)	98.6	1.0
			3	2.90 (± 0.15)	96.6	5.2
			1	0.96 (± 0.03)	96.0	3.1
		zg30	10	10.02 (0.15)	102	1.5
			5	5.32 (± 0.07)	106.4	1.3
			3	3.11 (± 0.04)	103.6	1.3
			1	1.22 (± 0.07)	122.0	5.7

In a study using the PULCON methodology, Watanabe et al. compared the results obtained with the ERETIC2 and the internal standard method using an NMR spectrometer equipped with 5mm diameter conventional probe in the quantification of the isolated okadaic acid compound. When the results of different integral regions in the spectra obtained from the same samples using zg pulse sequence are examined, the maximum RSD values calculated for the internal standard method and the ERETIC2 were reported as 5.16% and 3.77%, respectively (20). Using the ERETIC2 as a fast and precise method in addition to the gravimetric method for the quantitative determination of low molecular weight molecules in natural isolates and reference substances, Frank et al. used an NMR spectrometer with the conventional 5 mm diameter multinuclear probe hardware. They determined the accuracy of the ERETIC2 with the quantification of different concentrations of benzoic acid (1.72, 6.14, and 16.21 mM) and L-tyrosine (2.73, 7.33, and 9.99 mM) using 3.12 mM caffeine solution as their standard solution. Recovery values calculated for benzoic acid were 98.8%, 99.6%, and 99.2%, while recovery values calculated for L-tyrosine were 100.6%, 100.7%, and 100.9% (35). In another study, Selegato et al. evaluated the accuracy of the global spectral deconvolution-based NMR quantification method developed for the quantification of bioactive molecules in microbial cultures with the classical internal standard method and the ERETIC2. In this study, they used an NMR spectrometer equipped with a triple resonance CryoProbe. The reference calibration spectrum required for the ERETIC2 was obtained from the standard sucrose solution of 2 mM. In their study, the accuracy of the internal standard and ERETIC2 methodologies was determined by the quantification of standard benzyl benzoate solutions of different concentrations. The values obtained for 1 mM benzyl benzoate solution were reported as 0.96 mM for the classical internal standard method and 1.20 mM for the ERETIC2 (36).

In Table 1, it is clear that the quantification results obtained by using the HR-MAS probe exhibit lower RSD and better recovery compared to the literature provided above. This situation could be explained by the fact that the standard HR-MAS probes have higher radiofrequency (rf) field homogeneity compared to the conventional probes (34,37). Also, even if it was optimized for the experimental conditions, an HR-MAS probe properly placed into the NMR magnet can still have an inhomogeneous rf-field. This is a result of probe-independent factors such as the magnetic field induced by the non-spinning components of the NMR probe that are close to the sample, or the presence of temperature gradients within the sample caused by spinning and high radio frequency (38). However, rf-inhomogeneity will affect the internal standard method less than the quantification method based on an electronically generated reference signal (37). As a result, when Table 1 is examined, it is seen that DSS method provides a slightly lower RSD than ERETIC2 from the results obtained from both pulse programs. However, considering the possibility of internal standard substances to chemically interact with analyte molecules in the sample, we can conclude that the ERETIC2 is a more accurate approach for quantification.

Besides, it is an expected result that the accuracy of the results obtained from the same samples will be higher due to some errors that may occur during the preparation of different samples. Also, when the results obtained with both the DSS method and the ERETIC2 were examined, it was observed that the zg30 pulse sequence showed a shorter analysis time and consequently a faster quantification, but higher RSD and lower accuracy compared to the cpmgpr1d pulse sequence. The zg30 pulse sequence used in the quantitative NMR leads broad bands that complicate the integration of the signals in the NMR spectrum, and it decreases the signal-to-noise (S/N) ratio and increases the baseline distortions especially for low sample concentrations. Therefore, some signal resonances are overestimated in the zg30 pulse sequence (39). Additionally, suppressing the solvent resonance at very low sample concentrations with the appropriate pulse sequence allows the use of maximum receiver gain to improve the signal-to-noise (S/N) ratio of the spectrum and thus increases the accuracy of the quantitation results (10).

Metabolite concentrations calculated with ERETIC2

ERETIC2 has become a fast and reliable quantification method used in liquid-state NMR spectroscopy to determine the concentrations of target molecules in complex mixtures (35,40). The results obtained from this study showed that ERETIC2 method can be easily adapted to HR-MAS spectroscopy in determining metabolite concentrations of biological samples. Figure 3 shows the one-dimensional ^{1}H HR-MAS NMR spectrum obtained from human follicular fluid sample.

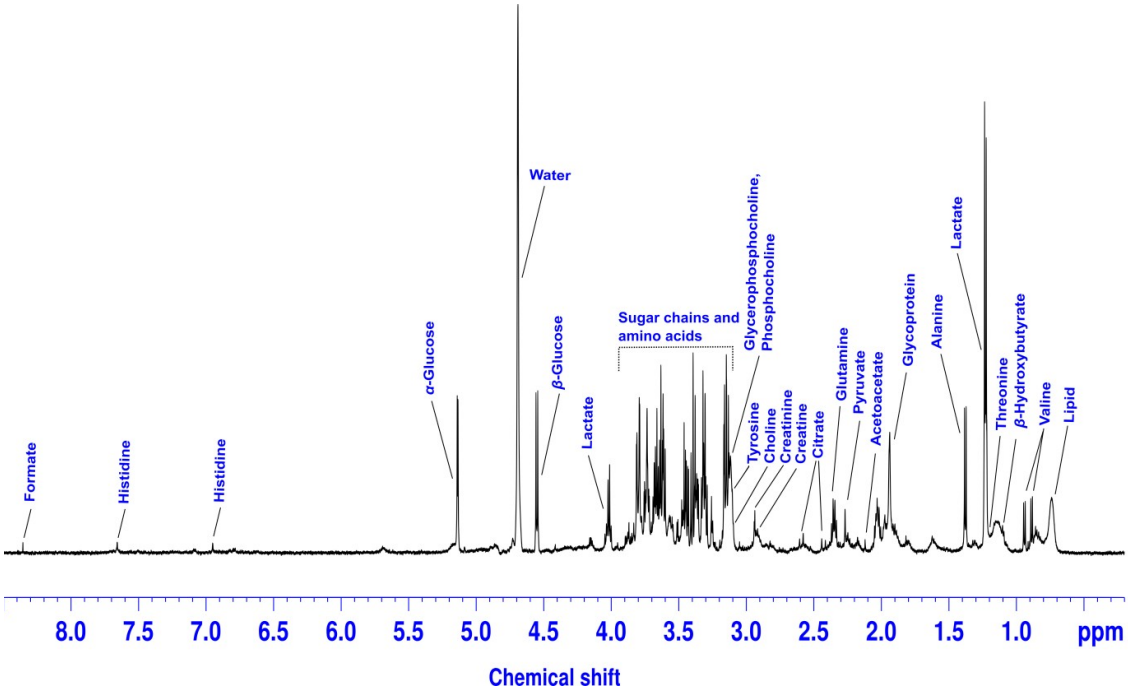

Figure 3: 600 MHz ^{1}H HR-MAS CPMG (Carr-Purcell-Meiboom-Gill) NMR spectrum recorded at a spin rate of 4 KHz and 295 K of human follicular fluid.

When Figure 3 is examined, it is seen that the signals of some metabolites were quite good resolved in the NMR spectrum of the follicular fluid. Thus, the integration of these metabolite signals can be easily performed. The concentration values of selected metabolites (valine, lactate, alanine, acetoacetate, pyruvate, glutamine, citrate, *β*-glucose, *α*-glucose) calculated using ERETIC2 method from ten different follicular fluid samples (from healthy control group and group with endometriosis) are given in Table 2. ^{1}H NMR experiments repeated three times with different samples obtained from each group. In Table 2, small standard deviation values obtained for the concentration results support the accuracy of

the method. Another method that can be used to evaluate the performance of ERETIC2 combined with HR-MAS spectroscopy in the quantification of follicular fluid metabolites may be to compare the results obtained in this study with those obtained from other methodologies. However, while studies using NMR spectroscopy in the precise quantification of the metabolite composition of the human follicular fluid are limited in the literature, relative quantitation is currently the focus of many NMR-based metabolomics studies (26,41).

Table 2. The absolute concentration values of the various metabolites contained in follicular fluids determined using the ERETIC2 method.

Metabolite	Integrated area (ppm)	Multiplicity	Group	Concentration (mM)	
				Healthy control Mean ± SD	Endometriosis Mean ± SD
Valine	0.90-0.87	doublet	γCH_3	0.23 ± 0.06	0.22 ± 0.08
Valine	0.95-0.91	doublet	$\gamma'CH_3$	0.17 ± 0.05	0.16 ± 0.05
Lactate	1.24-1.21	doublet	βCH_3	3.99 ± 1.60	5.90 ± 2.72
Alanine	1.39-1.35	doublet	βCH_3	0.52 ± 0.14	0.56 ± 0.16
Acetoacetate	2.13-2.10	singlet	γCH_3	0.07 ± 0.07	0.06 ± 0.02
Pyruvate	2.28-2.25	singlet	βCH_3	0.28 ± 0.06	0.30 ± 0.06
Glutamine	2.37-2.31	multiplet	γCH_2	0.87 ± 0.23	0.82 ± 0.30
Citrate	2.45-2.40	doublet	α, γCH	0.29 ± 0.12	0.29 ± 0.10
Citrate	2.61-2.56	doublet	α', γ'CH	0.34 ± 0.15	0.34 ± 0.11
Lactate	4.06-3.96	quartet	αCH	3.59 ± 1.38	5.19 ± 2.30
β-glucose	4.57-4.50	doublet	C^1H	1.64 ± 0.68	1.44 ± 0.97
α-glucose	5.17-5.09	doublet	C^1H	2.29 ± 0.71	2.07 ± 1.20

In the early studies, the researchers used different methods other than the NMR to determine the concentrations of metabolites such as glucose and lactate in follicular fluid, which were predicted to be important for oocyte quality. In their studies, Leese and Lenton calculated the glucose concentration in follicular fluid as 3.29 ± 0.09 mM and the lactate concentration as 6.12 ± 0.17 mM where they used an autoanalyzer for glucose and fluorometric method for lactate. They also reported the average pyruvate concentration calculated for some samples as 0.26 ± 0.008 mM (42). In another study, Gull et al. who investigated the glycolysis process in human ovarian follicles, determined the glucose and lactate concentrations in the follicular fluid using glucose analyzer and lactate spectrophotometric analyzer. They calculated the mean glucose and lactate concentrations in follicular fluid as 3.39 ± 0.91 mM and 3.17 ± 0.90 mM, respectively (43). Józvik et al. reported the glucose concentration as 2.78 mM in their studies by determining the sugar and polyol concentrations in a human follicular fluid using high-performance liquid chromatography (HPLC) method (44). Gosden et al. is the first group to use ^{1}H NMR spectroscopy to examine the molecular composition of follicular fluid. However, in their studies, they detected low molecular weight metabolites such as acetate, alanine, creatinine, glycine, D-3-hydroxybutyrate, lactate, and valine in the follicular fluid samples of some farm animals at concentrations exceeding 0.1 mM. In the quantitative determination of the molar concentrations of the metabolites, firstly, lactate concentrations were determined in each sample by an independent lactate dehydrogenase experiment. Next, these lactate values were used to determine metabolite concentrations by considering the NMR signals obtained from other metabolites (45). A detailed analysis of the metabolite composition of the human follicular fluid was first performed by Piñero-Sagredo et al. using a high-resolution liquid-state NMR spectrometer equipped with a 5 mm diameter triple CyroProbe. Although the concentrations of all metabolites determined in this study were not precisely calculated, a linear combination of reference NMR spectra obtained from solutions with known concentrations of both metabolites was used for glucose and lactate quantification. The glucose and lactate concentrations obtained by this method were reported as 2 mM and 5 mM, respectively (28). After Piñero-Sagredo and his group introduced a comprehensive metabolite profile of human follicular fluid into the literature, follicular fluid studies based on NMR spectroscopy focused on determining the relationship between follicular fluid metabolite composition and oocyte

development potential, embryo viability and etiology of diseases (46). Wallace et al. conducted ^{1}H-NMR based metabolomics analyzes using liquid-state NMR spectroscopy in follicular fluids collected from in vitro fertilization (IVF) patients. The collected samples were analyzed considering the number of embryo cells and noncleaving oocytes to test the ability of the follicular fluid metabolite composition in the prediction of successful IVF result. In Wallace's study, the metabolite concentrations were determined using a different method (amino acid analyzer) and the glucose concentration in the follicular fluid of two groups was reported as 3.25 ± 1.24 mM and 2.33 ± 0.57 mM (25). In another study, Karaer et al. conducted metabolomics analyzes using an NMR spectrometer with a conventional 5 mm diameter probe equipment to determine whether the change in the metabolite composition of follicular fluids collected from ovarian endometriosis patients was identifiable. In Karaer's study, ERETIC2 was used for the ^{1}H NMR-based quantitative analysis of metabolites in the women with ovarian endometriosis and healthy controls. The concentrations (mM) of different metabolites for each group were obtained as lactate (2.75 ± 1.1 and 1.6 ± 5.5), *α*-glucose (1.72 ± 0.5 and 1.25 ± 0.2), *β*-glucose (1.25 ± 0.2 and 0.83 ± 0.3), alanine (0.38 ± 0.11 and 0.27 ± 0.09), pyruvate (0.19 ± 0.04 and 0.14 ± 0.03) and valine (0.17 ± 0.03 and 0.12 ± 0.04) (27). When the results presented in Table 2 are examined, it is seen that lactate, alanine and pyruvate concentration values from the patient group were calculated higher than the healthy control group, similar to the results obtained by Karaer et al. (27). On the other hand, in contrast to Karaer et al., *α*-glucose and *β*-glucose concentration values were higher in the control group compared to the patient group. Although the relationship between the ratio of glucose and lactate concentrations and the anaerobic metabolism in the hyperstimulated follicle is out the scope of this study, in literature different values related to the ratio between glucose and lactate have been reported. In some studies, this ratio is given approximately 1:2 (42,28) while in others it is given approximately 1:1 (43,27). When the results obtained from this study are examined, it is seen that the ratio between total glucose and lactate is approximately 1:1 for healthy controls and approximately 1:2 in the patient group. It has been shown that, there is a negative correlation between follicle size and glucose concentration and a positive correlation between follicle size and lactate concentration (43). In this study, the glucose and lactate concentration values calculated from follicular fluid samples collected from follicles with a diameter of about 20 mm are compatible with the concentration values obtained from the follicles of the same size by Gull et al. (43).

CONCLUSION

NMR spectroscopy has a very important place among quantification methods, as it provides a direct relationship between peak area and absolute concentration as well as providing valuable structural and diagnostic information within short analysis times. Recent advances in PULCON methodology have shown that analyte spectra used in quantitative NMR can be obtained completely without internal standard reference material. In this context, ERETIC2 can be easily applied in determining metabolite concentrations in intact biological fluids. Also, attention should be paid to the use of the same pulse sequence in the analysis of the known reference solution and the subsequent analyte solution, the effects of factors such as solvent and concentration on the acquisition parameters such as relaxation delay and pulse length, and the salt concentration contained in the sample when obtaining the reference spectrum for the ERETIC2 method. Although HR-MAS NMR spectroscopy is generally used in the analysis of heterogeneous sample systems, it can be an excellent alternative in the analysis of limited amounts of biological fluid samples and quantification of unknown species in these samples. Sample leakage that may occur in the rotor system is one of the most important factors that can lead to quantitation errors in HR-MAS studies of biological fluids. However, these errors can be prevented with care and attention to be shown during rotor assembly. Additionally, it should be noted that sample recovery in HRMAS studies is difficult. When the results obtained in this study and previous literature data obtained from other methodologies are compared, it is seen that the ERETIC2 method combined with HR-MAS NMR spectroscopy is reliable and accurate in determining follicular fluid metabolite concentrations.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the author.

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Supporting Information

A simple and feasible method for the quantification of metabolites in the human follicular fluid using ^{1}H HR-MAS NMR spectroscopy

Akın MUMCU[1,2*]

[1]Laboratory of NMR, Scientific and Technological Research Centre, Inonu University, Malatya, Turkey
[2]Reproductive Sciences & Advanced Bioinformatics Application & Research Centre, Inonu University, Malatya, Turkey

Table S1: Concentration values obtained as a result of two different methods and 5 different measurements for same standard creatine solutions at different concentrations. Concentrations of creatine in solutions were calculated from the 9 proton singlet signals of DSS in the range of 0.02 to - 0.17 ppm and the 3 proton methyl signal of creatine in the range of 3.05 - 2.9 ppm using equation 1 and 2.

Method	Std creatine solutions (mM)	Concentration results obtained using two different pulse programs (mM)									
		cpmgpr1d					zg30				
		Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
DSS method	10	9.80	9.80	9.78	9.77	9.81	10.80	10.81	10.80	10.82	10.79
	5	4.97	5.08	5.03	4.96	4.94	5.47	5.39	5.48	5.37	5.50
	3	2.91	2.89	2.90	2.90	2.95	3.40	3.42	3.44	3.42	3.44
	1	0.98	0.99	0.98	0.97	0.98	1.25	1.28	1.27	1.24	1.31
ERETIC 2	10	9.57	9.53	9.50	9.45	9.51	10.31	10.22	10.18	10.13	10.18
	5	4.98	4.97	5.10	4.86	4.82	5.29	5.27	5.36	5.20	5.29
	3	2.71	2.66	2.66	2.76	2.73	3.12	3.11	3.13	3.12	3.12
	1	0.97	0.94	0.92	0.91	0.92	1.14	1.20	1.18	1.17	1.19

Table S2: Concentration values calculated using two different methods as a result of NMR measurements obtained from 5 different samples prepared from each standard creatine solution at different concentrations. In concentration calculations, 9 proton singlet signals of DSS in the range of 0.02 to - 0.17 ppm and 3 proton methyl signal of creatine in the range of 3.05 - 2.9 ppm were used.

Method	Std creatine solutions (mM)	Concentration results obtained using two different pulse programs (mM)									
		cpmgpr1d					zg30				
		Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
DSS method	10	9.80	9.97	9.99	9.79	9.69	10.80	10.79	10.81	10.81	10.80
	5	4.97	4.81	4.94	4.89	4.98	5.39	5.42	5.44	5.51	5.46
	3	2.90	2.94	2.96	2.97	2.99	3.47	3.40	3.46	3.43	3.45
	1	0.98	1.02	1.01	1.02	1.03	1.25	1.30	1.30	1.32	1.23
ERETIC 2	10	9.57	9.51	9.44	9.15	9.47	10.18	9.94	10.13	9.80	10.04
	5	4.98	4.91	4.93	4.97	4.86	5.27	5.41	5.29	5.39	5.26
	3	2.76	2.73	3.08	2.96	2.98	3.04	3.12	3.12	3.14	3.12
	1	0.92	1.00	0.95	0.97	0.97	1.14	1.32	1.23	1.24	1.19

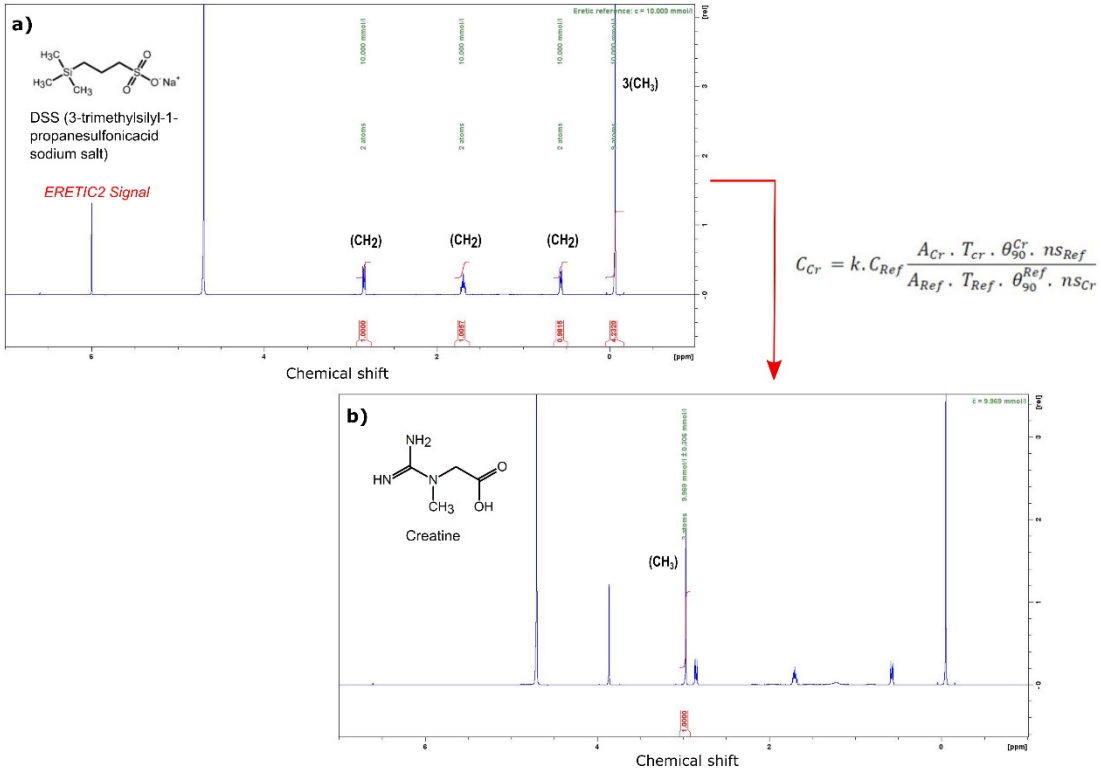

Figure S1: Quantification procedure used for the ERETIC2 a) one dimensional ^{1}H HR-MAS NMR spectrum of a 10 mM DSS solution (calibration step where the ERETIC2 signal is generated using DSS sample). To determine the 90° pulse (Θ_{90} = 5.75 μsec), the command *pulsecal* was used, where relaxation delay was 10 s; number of experiments was 25; and the pulse calibration was performed in 0.5 μsec interval within 2 μsec. b) ^{1}H HR-MAS NMR spectrum of 10 mM creatine solution containing internal standard (quantification step in which the concentration of standard creatine solutions in different concentrations is determined). The acquisition parameters given above were used to determine the 90° pulse (Θ_{90} = 6.0 μsec). Besides, The FIDs were processed by multiplying with 0.3 Hz line broadening parameter. NMR spectra were phase-corrected manually and the "absn" function was used for the baseline correction.

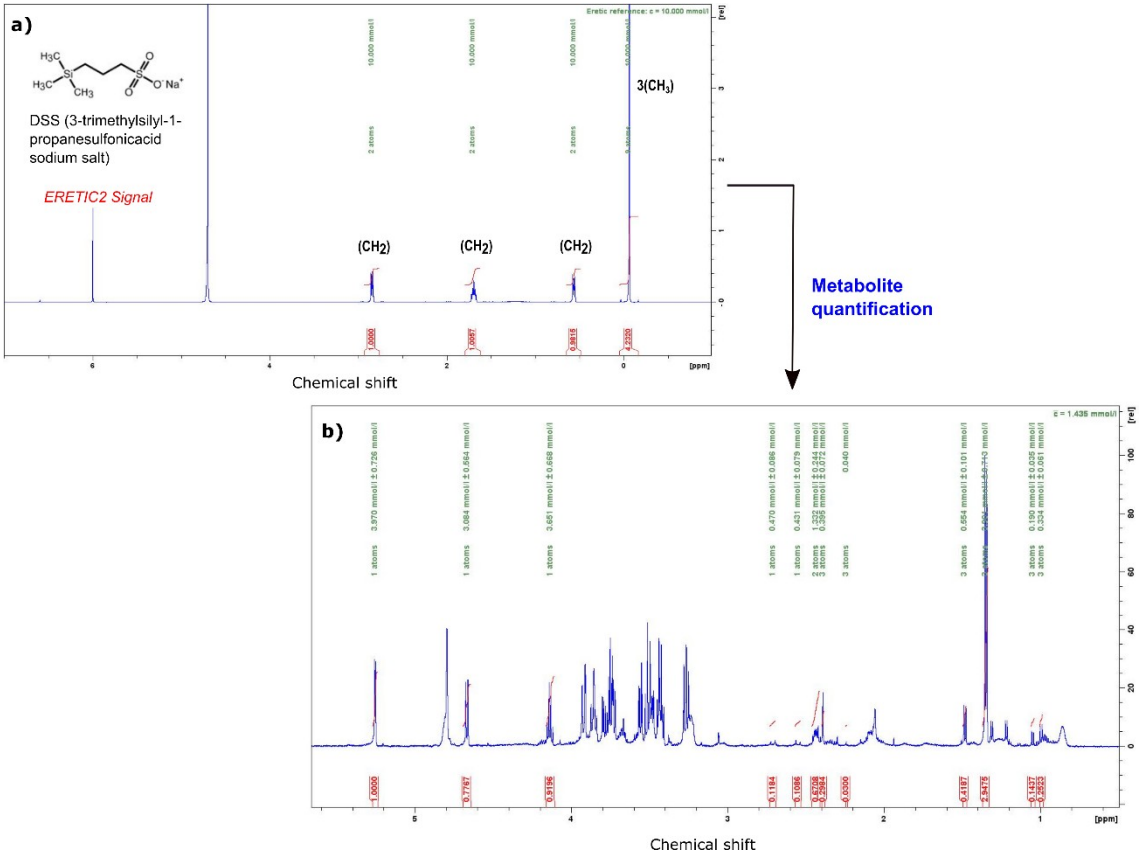

Figure S2: Determination of follicular fluid metabolite concentrations (mM) using the ERETIC2 a) one dimensional ^{1}H HR-MAS NMR spectrum of a 10 mM DSS solution (calibration step where the ERETIC2 signal is generated using DSS sample). b) representative ^{1}H HR-MAS NMR spectrum of the follicular fluid sample (the relevant metabolite concentrations were determined by means of equation number 2).