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Semi-parametric time-domain quantification of HR-MAS data from prostate tissue

Helene Ratiney^{b,*†}, Mark J. Albers^{a,†}, Herald Rabeson^b, and John Kurhanewicz^a

^a Department of Radiology, University of California - San Francisco, San Francisco, CA, USA

^b Laboratoire CREATIS-LRMN, CNRS UMR 5220, Inserm U 630, Insa-Lyon, Université de Lyon, Villeurbanne, France

Abstract

High Resolution – Magic Angle Spinning (HR-MAS) spectroscopy provides rich biochemical profiles that require accurate quantification to permit biomarker identification and to understand the underlying pathological mechanisms. Meanwhile, quantification of HR-MAS data from prostate tissue samples is challenging due to significant overlap between the resonant peaks, the presence of short T_2^* metabolites such as citrate or polyamines (T_2 from 25 to 100 msec) and macromolecules, and variations in chemical shifts and T_2^* s within a metabolite's spin systems. Since existing methods do not address these challenges completely, a new quantification method was developed and optimized for HR-MAS data acquired with an ultra short T_E and over 30,000 data points. The proposed method, named HR-QUEST (High Resolution – QUEST), iteratively employs the QUEST time-domain semi-parametric strategy with a new model function that incorporates prior knowledge from whole and subdivided metabolite signals. With these features, HR-QUEST is able to independently fit the chemical shifts and T_2^* s of a metabolite's spin systems, a necessity for HR-MAS data. By using the iterative fitting approach, it is able to account for significant contributions from macromolecules and to handle shorter T_2 metabolites, such as citrate and polyamines. After subdividing the necessary metabolite basis signals, the root mean square (RMS) of the residual was reduced by 52% for measured HR-MAS data from prostate tissue. Monte Carlo studies on simulated spectra with varied macromolecular contributions showed that the iterative fitting approach (6 iterations) coupled with inclusion of long T_2 macromolecule components in the basis set improve the quality of the fit, as assessed by the reduction of the RMS of the residual and of the RMS error of the metabolite signal estimate, by 27% and 71% respectively. With this optimized configuration, HR-QUEST was applied to measured HR-MAS prostate data and reliably quantified 16 metabolites and reference signals with estimated Cramér Rao Bounds $\leq 5\%$.

Keywords

Time-domain quantification method; high-resolution magic angle spinning; prostate tissue sample; metabolite basis set; macromolecules

*Correspondence to: H. Ratiney, Laboratoire CREATIS-LRMN, CNRS UMR 5220, Inserm U630, Université Claude Bernard Lyon 1, CPE, Domaine Scientifique de la DOUA, 3 rue Victor Grignard, Aile C, 69616 Villeurbanne, France.

†Co-first Authors.

INTRODUCTION

High Resolution – Magic Angle Spinning (HR-MAS) spectroscopy provides a technique to metabolically characterize a variety of biological samples, such as surgical specimens, cell culture samples, or biopsy samples, in a non-destructive manner (1–10). With HR-MAS, it is possible to obtain immunohistochemical, genetic or pathologic data from the same sample following the experiment (2,3,6,11–13). While the combination of metabolic and biological data can help elucidate and characterize new biomarkers, the HR-MAS protocol introduces extra challenges when analyzing the metabolic information. In particular, the HR-MAS experiments must employ a relatively low spin rate to preserve the metabolic and structural integrity of the sample and employ an ultra-short echo time (T_E) for prostate tissue samples. The lower spin rate produces slightly larger spinning side bands, which requires a larger spectral bandwidth and more data points to prevent the side bands from wrapping back into the primary spectrum. Moreover, accurate quantification of citrate and polyamines, two key prostate metabolic makers with short T_2 s requires the use of ultra-short T_E which yields spectra with significant contributions from macromolecules and mobile lipids. Additionally, the high spectral resolution of the experiments, especially at high field strengths, permits the detection of variation in chemical shift (δ_s) and T_2^* between different spin systems within the same molecule, which are likely caused by changes in the metabolite's microenvironment (14–16). Therefore, for prostate tissue data, the HR-MAS quantification algorithm must be able to process spectra with many data points, to separate the macromolecules from the metabolites, and to independently fit the δ_s s and T_2^* s of a metabolite's spin systems. Above all, the quantification algorithm also needs to resolve the overlapping resonances produced by numerous metabolites that are present in biological spectra and should be relatively automatic so as to minimize any user dependent bias.

Several different approaches have been proposed for analyzing the metabolites found in HR-MAS data. One approach combines the spectral quantification and statistical analysis into one process by using Principal Component Analysis to directly analyze each spectral point and to identify the main differences between groups of data. While this technique is very effective at classifying the HR-MAS spectroscopy data into groups (8,17–19), it does not allow for quantitative identification of a specific metabolite. As a result, it is more difficult to investigate the underlying pathological and biochemical mechanisms, to identify new biomarkers, and to translate the findings into the clinic. Another class of quantification techniques computes the area of the resonance peaks by using peak integration or Lorentzian-Gaussian peak fitting (3,4). These techniques are widely available and are well suited for quantifying completely resolved and unobstructed resonances. However, peak integration and peak fitting are not ideal for quantifying prostate HR-MAS spectra because its constituent metabolites and macromolecules are not completely resolved. The last class of quantification techniques are fitting procedures that employ the prior knowledge contained in a metabolite basis set to measure the concentrations of metabolites with overlapping spectra in either the frequency or time domain (20–22). Many of these techniques (LCModel, AQSES, QUEST) are capable of quantifying short echo time data that contain significant amounts of macromolecules and have been used to quantify *ex-vivo* high resolution spectroscopic data (7,23–27). However, none of these techniques address variations in chemical shifts and T_2^* within a molecule's spin systems, which induces mismatches between the metabolite basis set and the biological sample data. A recently developed *ex-vivo* NMR quantification algorithm models these variations but its filtering of macromolecules is insufficient for prostate tissue HR-MAS data (24).

The goal of this project was to develop a quantification algorithm for HR-MAS data from prostate tissue samples that satisfies all of these needs. The resulting algorithm is based on the semi-parametric time-domain approach used by QUEST to quantify *in-vivo* short T_E data

acquired at low to medium field strengths (28). QUEST attempts to separate the metabolites from the macromolecules and mobile lipids by ignoring the first points of the FID and parametrically quantifying the metabolites using a basis set. Then it non-parametrically models the macromolecules present in the remaining signal with an Hankel Lanczos Singular Value Decomposition (HLSVD) and ultimately estimates the metabolites from a background free FID. Recently, QUEST was able to quantify the metabolites in short T_E HR-MAS data from brain tissue, which did not contain citrate nor polyamines and had low macromolecule levels, after its spectral bandwidth was reduced by filtering(23). In the algorithm presented here, called HR-QUEST (High Resolution – QUEST), the model function and constraints employed permit independent fitting of the frequency offset, extra damping, and/or amplitude for sub-components of a metabolite. The separation of the macromolecule and metabolite signals was improved by iteratively estimating the metabolites and modeling the macromolecules and by incorporating the longer T₂ macromolecules into the basis set. Monte Carlo studies were conducted to determine benefits of these two improvements. Quantifications of measured HR-MAS data from prostate tissue samples are also presented and analyzed.

METHODS

HR-QUEST algorithm

In order to quantify HR-MAS data acquired from prostate tissue under conditions that preserve the tissue for subsequent analyses, HR-QUEST was designed to analyze up to 32,768 complex points from each HR-MAS FID using a basis set that contained up to 55 elements. HR-QUEST offers new flexibility when fitting HR-MAS data while remaining easy-to-use by defining default values for all the customizable parameters. It was configured to use a command-line interface and a grid computing environment, which allowed for simultaneous processing of several data sets. This new algorithm is available to the public upon request from the corresponding author. Algorithmically, HR-QUEST uses a Non-Linear Least Square (NLLS) minimization and a basis set to parametrically estimate the metabolite signal in the time domain and uses an HLSVD to model the background. A new model function and constraints were introduced that handle intra-molecular chemical shift variations and allow for individual phase estimation of basis elements. This latter feature was essential for accurate quantification of the Electronic REference To access In vivo Concentrations (ERETIC) signal because it was often slightly out of phase from the metabolites.

As in QUEST, the HR-MAS complex time domain data points $\hat{x}_n$ are modeled as a linear combination of M weighted metabolite signals, referred to as the basis set. HR-QUEST expands the model function for $\hat{x}_n$ to allow each metabolite signal to be split into I_m sub-components. Thus, the model function of HR-QUEST can be expressed as:

$$\hat{x}_n = \exp[j\varphi_o] \sum_{m=1}^M \sum_{i=1}^{I_m} \Delta a_{m,i} \hat{c}_{n,m,i} \exp \left[(\alpha_{m,i}^{sv} + \Delta \alpha_{m,i} + j2\pi \Delta f_{m,i}) t_n + j \Delta \varphi_{m,i} \right] \quad [1]$$

where n is the time index, t_n is the time in seconds. m is the basis set index and ranges from 1 to M . If the m^{th} metabolite is divided into I_m sub-components, then i is the sub-component index. $\hat{c}_{n,m}$ is the n^{th} complex-valued time-domain data point of the m^{th} metabolite signal, which can be split into I_m sub-components. For convenience, the subscript i will not be included in the notation going forward. α_m^{sv} is a fixed apodization factor that can be applied prior to the fit procedure enabling the adjustment of the starting value (introducing the SV superscript) for an individual basis element. Δa_m , $\Delta \alpha_m$, Δf_m , and $\Delta \varphi_m$ are the amplitude

weights, extra damping / apodization factors, frequency offsets, and phase adjustments, respectively, that HR-QUEST can estimate for each basis element. φ_0 is the zero order phase. HR-QUEST estimates all of the requested parameters by applying a NLLS minimization to the sum of the square of the residual. Prior knowledge can be incorporated into the fitting in a manner similar to the AMARES algorithm (29) by instructing HR-QUEST to constrain $\Delta a_{m,i}$, $\Delta f_{m,i}$, and/or $\Delta a_{m,i}$ to be the same for multiple elements of the basis set, within a metabolite or between metabolites. This new capability reduced the number of parameters estimated by HR-QUEST and prevented HR-QUEST from producing a physically impossible solution when a metabolite was split into I_m sub-components. User-definable soft constraints are placed on Δa_m and Δf_m , which restricts these values to a limited range. For this study, Δa_m and Δf_m were restricted to 0 – 12 Hz and –9 – +9 Hz, respectively, and φ_0 was not estimated.

The separation of the background and the metabolites was improved by iteratively estimating the metabolites and the background K times as shown in Figure 1.

For the first iteration, HR-QUEST performs an NLLS minimization on the data after omitting the first n_X points of the FID, where n_X is specified by the user, and then estimates the background using an HLSVD to model the remaining signal as shown in Figure 1.

During each successive iteration, n_X is progressively reduced until zero points are omitted for the last iteration. HR-QUEST allows the user to define K. Additional commands that control the HLSVD modeling were added to HR-QUEST and are described in the Appendix.

Estimated Cramér–Rao Bounds (eCRBs) were computed for the metabolite amplitudes using the final fitting parameters and an estimate of the standard deviation (SD) of the noise (σ). In an attempt to incorporate the error caused by a poor fit into the CRBs, σ is calculated using the first n_σ points of the time domain residual instead of the last points. n_σ should correspond to the time at which the HR-MAS time-domain signal has significantly decayed, which can be approximated by two times the T_2^* of the metabolite signal with a high SNR and the longest T_2^* (e.g Choline). n_σ was set to 3,512 points for the present studies.

Using HR-QUEST to quantify prostate HR-MAS data

Several of the metabolites in the basis set were sub-divided into two or more basis set elements based on visual observations that one or more of their spin systems had a different δ_s and/or T_2^* from the molecule's other ^1H 's. Table 1 specifies how the various metabolites were divided and which sub-components were constrained to have the same $\Delta a_{m,i}$ and $\Delta f_{m,i}$ for a given metabolite, m . All $\Delta a_{m,i}$ s were constrained to be the same. Several of the sub-component basis elements were created by using the HLSVD in jMRUI to model the relevant portion of the measured basis spectrum (30). Additionally, the lactate C-2 ^1H 's were frequency shifted by 6.5 Hz to match the δ_s s observed in the tissue spectra. Similarly, the citrate CH_A and CH_B protons were shifted by –4.6 and 12.5 Hz, respectively, the glutathione C-4 ^1H 's by 3 Hz, and the phosphoethanolamine C-1 and C-2 ^1H 's by 2.5 and 3.7 Hz, respectively. Also, the α_m^{sv} for both sub-components of citrate was set to 4 Hz.

Employing the features available in HR-QUEST, the background modeling was refined to reduce the error in the metabolite estimation and the residual. These improvements were accomplished by evaluating different values for K (the number of iterations), by partially parameterizing the background estimation, and by increasing n_X to 80 points (which corresponded to a ~91% decay in the background signal). When different values of K were evaluated, the entire background signal was approximated with a mathematical function determined by an HLSVD that had no physical meaning. This non-parametric modeling will

be referred to as the “HLSVD only” fits. For the “partial parameterized” fits, the macromolecules with longer T_2^* s (narrower linewidths) were estimated by adding them to the basis set. Therefore, these components were parametrically estimated by the NLLS minimization and the remaining broad background was non-parametrically estimated by the HLSVD. To obtain the longer T_2 macromolecular basis elements, the background signals reported by HR-QUEST for a separate set of HR-MAS data from prostate tissue samples were averaged and modeled using HLSVD. The eight components shown in Figure 2 were isolated from the HLSVD results and incorporated into the basis set. During the fitting, HR-QUEST estimated the phase for the macromolecule peaks at 1.3 and 2.3 ppm.

HR-MAS protocol and HR-QUEST basis set

Data were acquired at 11.7 T using a Varian INOVA spectrometer equipped with a 4-mm gHX nanoprobe. The HR-MAS data were acquired using previously published methods that preserve the tissue for subsequent genetic and/or histological analysis and ensure accurate detection of key prostate metabolites with shorter T_2 s, such as Citrate and Polyamines (4,31). In brief, the samples were placed inside of wide-mouth zirconia rotor (31), weighed, spun at 2,250 Hz, and maintained at approximately 1°C during the experiments. For the basis spectra, a pulse-and-acquire sequence ($TE = 0$) was used to record 40,000 complex data-points during 2 seconds and 64 transients with a 20 kHz spectral bandwidth, 6 sec delay, 2 sec water presaturation pulse ($TR = 10$ sec), and 90° excitation pulse. An electronic quantification reference was added using the ERETIC method with an 18 dB pulse power (31–33). For the tissue samples, the data acquisition was adapted to include 128 transients, 0 sec delay, and an ERETIC power of 0 dB.

For the HR-QUEST basis set, a solution spectrum was acquired for each metabolite listed in Table 1 using a highly concentrated solution (8–98 mM) that was buffered to a pH of 7.3 using potassium phosphate and titrated when necessary. Measured basis FIDs were also included for glucose, TSP, and ERETIC. Simulated basis FIDs were included for several small, unidentified peaks at 2.04, 2.23, 2.42, and 3.20 ppm and beta-hydroxybutyrate. Since the spermidine and spermine spectra are highly dependent upon their microenvironment (14), a simulated Gaussian peak was added to the basis set at 3.15 ppm to correct for discrepancies between polyamines in the basis set and the tissue data. For citrate, the basis FID was acquired from a 50 mM solution that contained 11 mM ZnCl₂, which produced a J-coupling of 15.7 Hz at 11.7T. This solution was designed to mimic the average J-coupling observed for citrate in prostate tissue samples analyzed at 11.7T.

Evaluating the HR-QUEST optimizations

The new features of HR-QUEST were evaluated using HR-MAS data from prostate tissue and Monte Carlo simulations. After obtaining approval from our institution’s Institutional Review Board and securing informed consent from each patient, prostate tissue samples were obtained from 11 patients that underwent radical prostatectomy surgeries and processed using a previously published protocol (2). All the samples were histologically classified as normal glandular prostate tissue by an experienced pathologist. Three samples were from patients that were previously treated with Lupron, yielding spectra without citrate and polyamines (34). These samples will be referred to as the “therapy samples” and permitted evaluation of the HR-QUEST fitting in spectra without the short T_2^* metabolites. The remaining samples had ample amounts of citrate and polyamines and will be referred to as “normal samples”. For each sample, HR-MAS data was acquired using the protocol described in the previous section.

Each data set was prepared for quantification by linear predicting the first two points of the FID using Advanced Chemistry Development Labs’ 1D NMR processor. Next, the data was

phased and frequency referenced to the CH₃ peak of creatine, and the residual water peak was removed using jMRUI (30). Then the data was quantified with HR-QUEST using the configuration necessary to evaluate the various modifications and optimizations made to the algorithm.

The benefits of splitting some of the basis elements into multiple sub-components was assessed by fitting the prostate tissue samples with HR-QUEST using 2 iterations and a basis set with and without some of the elements split. The split elements were restricted by the constraints listed in Table 1. The root mean square (RMS) of the time-domain residual was computed for both cases and compared. Also, the difference in $\Delta f_{m,i}$ for a given metabolite's sub-components were computed on the final HR-QUEST fits for each tissue sample if the metabolite's eCRBs was $\leq 5\%$. The average and range of this difference is reported in Table 1.

A Monte Carlo study was created to evaluate the benefits of increasing K and of partially parameterizing the macromolecules present in the data. In an attempt to mimic the real data, the simulations consisted of 100 data sets generated by adding randomly chosen Gaussian noise, a randomly varied background signal, and the de-noised version of the residual from a representative tissue sample to a reference signal (see Appendix for details). The reference metabolite signal was created by weighting the basis set with the $\Delta a_{m,i}$, $\Delta \alpha_{m,i}$ and $\Delta f_{m,i}$ results obtained from the same representative sample. Each simulation was fit with HR-QUEST using 2, 6, and 12 iterations, with and without the partially parameterized macromolecules included in the basis set. The simulations were summarized by computing the mean and standard deviations of the RMS of the residuals and the RMS errors (RMSE) described in the Appendix.

The HR-QUEST quantification accuracy and reproducibility were evaluated on the prostate HR-MAS data using 2 and 6 iterations and with and without the partially parameterized macromolecules. This analysis consisted of examining the remaining signal in the residual, comparing the mean and variability of the background signal, and calculating the mean eCRBs. For the residual signal analysis, the absolute value of the residual spectra were computed for all 11 prostate samples and averaged together for the 4 scenarios mentioned above. The background spectra were compared by normalizing HR-QUEST's background estimates from each tissue sample to correct for differences in the sample volume and other sample-to-sample differences. The normalized background spectra were plotted for the 4 scenarios along with the averaged background spectrum for each scenario.

RESULTS

Benefits of splitting metabolite basis spectra

The results in Table 1 and Figure 3 demonstrate the importance of splitting some of the basis spectra into sub-components and allowing HR-QUEST to independently fit the frequency shift and damping factors for the sub-components when necessary. The difference in the $\Delta \alpha_{m,i}$ s for unconstrained sub-components had a range of 12.6, 4.5, 1.8, and 1.6 Hz for glutathione, citrate, myo-Inositol, and lactate, respectively. The $\Delta f_{m,i}$ s were different for sub-components from the same metabolite and the difference varied from sample to sample as demonstrated by the Range of $\Delta f_{m,i}$ Difference column in Table 1. For example, the A and B protons of citrate had $\Delta f_{m,i}$ s of -9.1 and 7.8 Hz, respectively, and, the difference in these two offsets varied by 3.02 Hz. By splitting the citrate spectrum and adding the appropriate fitting constraints, HR-QUEST was able to correctly fit the data despite the sample-to-sample variations in the citrate δ_s and Δ (35). Similar benefits were obtained for phosphoethanolamine and lactate, which had Range of $\Delta f_{m,i}$ Differences of 3.05 and 2.06 Hz, respectively. As an aside, the chemical shifts of the majority of the metabolites varied

more across the samples when they were referenced to TSP as opposed to creatine as demonstrated by a 1.53 Hz reduction in the average frequency offset range from 2.55 to 1.02 Hz. However, HR-QUEST was able to fit the HR-MAS data even when the data was referenced to TSP.

Figure 3A shows that the RMS of the residual was reduced for all the prostate tissue samples after splitting the metabolite basis signals. On average, the residual was reduced from $3.48 \times 10^4 \pm 1.67 \times 10^4$ to $1.65 \times 10^4 \pm 4.60 \times 10^3$ a.u., $p < 0.01$. The tissue samples from the therapy patients also benefited from the splitting of the basis elements but the reduction in the residual was less ($1.85 \times 10^4 \pm 4.32 \times 10^3$ to $1.45 \times 10^4 \pm 2.48 \times 10^3$ a.u.) because the samples did not contain citrate nor polyamines. A representative example of the fitting from a normal tissue sample obtained before and after the basis spectra were split, shown in Figures 3B and 2C, illustrates that splitting was essential for citrate (2.55 & 2.71 ppm) and lactate (1.34 & 4.13 ppm). In addition, there was a noticeable reduction in the frequency domain residual for the polyamines (1.77, 2.09 & 3.12 ppm), glutamate (2.10, 2.35 & 3.77 ppm), glutamine (2.13, 2.45, & 3.77 ppm), and phosphoethanolamine (3.98 ppm).

Monte carlo simulation results

The fitting was further improved by increasing the number of iterations used for the fitting. With 6 iterations, HR-QUEST required ~20 min to quantify one FID on a 2.2 GHz CPU with 4 GB of RAM. The overall RMSE of the estimated background and metabolite FIDs from the Monte Carlo simulations with 6 iterations were 29 and 23% lower, respectively, than with 2 iterations as shown in Figure 4A. Furthermore, the RMSE of glutathione, glutamate, alanine, glycine, phosphoethanolamine, glutamine, and glucose concentrations were reduced by between 4.5 and 48% with 6 iterations as shown in Figure 4B. For some metabolites, the RMSE increased when more iterations were added (right side of Figure 4B). However, the partial parameterization of the macromolecules typically more than compensated for this degradation in RMSE (Figure 4C). While the overall RMSE for 12 iterations was comparable to 6 iterations, the RMSE for several metabolites was significantly worse at 12 iterations. When the background estimation was partially parameterized, the improvement in the overall RMSE with 6 iterations was less, but it was still improved.

The overall RMSE for the metabolite signal and the background signal were reduced by 63% and 67%, respectively (Figure 4A), when adding the 8 macromolecule peaks shown in Figure 2 to the basis set and using 6 iterations for the fitting. The reduction in the RMSE for the individual metabolites was largest in the Monte Carlo simulation for glutamine, glucose, glutathione, glutamate, and alanine, which experienced a >5% reduction in RMSE with the partially parameterized macromolecules and 6 iterations (Figure 4C). Except for lactate, all the other metabolites demonstrated a more modest reduction in RMSE (<5%). The increase in the RMSE for lactate was <1% and was considered insignificant given the improvement in the RMSE for the other metabolites. Excluding glucose and glutamine, the RMSE of the individual metabolites was less than 10% after adding the macromolecule peaks, with the majority being <5% (Figure 4C). Most of the metabolites with a RMSE >5% exhibited a low SNR in the reference spectrum because of the low concentration found in the sample spectra.

Improvements in tissue sample fits

Reviewing the fits for the representative therapy sample and the representative normal sample shown in Figure 5, the additional iterations and partial parameterization reduced the residual at 1.20–1.32, 1.36–1.57, 1.67–1.80, 1.88–2.39, and 4.16–4.35 ppm as indicated by the highlighted regions. The same improvements were observed in the other prostate tissue

samples as well (Figure 6C). The fitting enhancements also significantly reduced the residual signal at 3.23 ppm observed in the therapy samples. This residual was caused by incorrect fitting of the large PC and GPC peaks present in these samples. While the height of the residual in the frequency domain near lactate at ~1.33ppm was rather large, the ratio of the residual's integral in this region to the area of the lactate doublet was 0.45% for the example in Figure 5A. Therefore, the error in the lactate estimate resulting from this residual was insignificant. Similarly, while the height of the residual in the frequency domain was greater than the noise at some downfield frequencies, the net signal was near zero indicating that the impact on the metabolite estimations was negligible.

Figure 2 illustrates that the parameterized macromolecules modeled most of the background components with narrower linewidths. The fit of the parameterized macromolecule peaks occasionally produced individual spectral components with some dispersive character. However, the final background estimate was not dispersive because the HLSVD modeled component of the background mathematically compensated for the dispersive character exhibited by some of the fitted macromolecule basis signals.

Furthermore, the parameterized peaks at 1.4, 2.05, 2.3, 3.0 and 3.3 ppm produced more pronounced peaks in the average baseline estimation shown in Figure 6 and were evident in the representative examples in Figure 5 as well. Consistent with the Monte Carlo simulations, these improvements affected the fits for alanine, glutamate, glutamine, creatine, choline, phosphocholine, glycerophosphocholine, taurine, myo-inositol, and the polyamines, as well as possibly affecting the lactate fit.

Reliability of the tissue sample fits

The normalized background estimates in Figure 6 demonstrate that the partial parameterization provided better separation of the background and polyamines at 3.1 ppm as shown by the reduction in the background signal at 3.1 ppm. Interestingly, simply increasing the number of iterations to six also reduced the background signal at 3.1 ppm. The normalized background estimates also exhibited a reduction in the variation across the 11 prostate samples near glutamate C-4 and Citrate when partially parameterizing the background. After the partial parameterization, the average of the SD of background estimates from 2.3–2.9 ppm was reduced by 37 and 10% (2 and 6 iterations, respectively) relative to the pure HLSVD modeling.

The eCRBs from the partially parameterized fits with 6 iterations were generally quite small for most of the metabolites. Based on this data, metabolite amplitudes with eCRB percentages $\leq 5\%$ were considered to be reliable. eCRBs $> 5\%$ and $\leq 10\%$ were considered marginally reliable, and those $> 10\%$ were deemed unreliable. In the end, the estimation of glucose, glutamine, and ethanolamine were considered to be unreliable on average because their eCRBs were $> 10\%$. Each of these metabolites had very low SNR (below a factor of four in the frequency domain) in the sample spectra on average. Phosphocholine's estimates were more marginal on average. Ultimately though, a metabolite estimate's reliability must be evaluated on a sample-by-sample basis. For example, the eCRB for phosphocholine was $> 5\%$ in some samples because its SNR was very low in those samples.

DISCUSSION

A new method, HR-QUEST, was developed that provides a semi-automated approach for quantifying the metabolite levels in high-resolution FIDs from intact prostate tissue samples. With HR-QUEST, the user can choose to constrain the frequency offset, extra damping, and/or amplitude for different elements of the basis set, allowing HR-QUEST to account for differences in the δ_s s and T_2^* s between a metabolite's spin systems. Furthermore, HR-

QUEST's iterative estimation of the metabolites and background, combined with the parameterization of the macromolecules with longer T_2^* s, produced a more accurate and reliable fit of the prostate tissue's HR-MAS spectra. In its final form, HR-QUEST reliably quantified 16 metabolites and reference signals in the HR-MAS spectra included in this study, with estimated Cramér Rao Bounds below 5%.

For several metabolites, the δ_s s observed in the tissue were different from those observed in solution at physiologic pH. Furthermore, the deviation in the δ_s was often unique to a spin system within a given metabolite and unique to a particular sample. In some cases, the extra damping (T_2^*) was also spin system specific. These observations are consistent with studies showing that some metabolites' δ_s s, J-couplings, and T_2^* s are dependent on the metabolites' microenvironment, in particular its pH and ion concentration (14,16,35,36). While these variations are relatively small, they impact the quantification of high-resolution spectra. The ability to constrain parameters between basis elements allowed HR-QUEST to account for variations in δ_s s and T_2^* s within a molecule and across samples. As an added benefit, the constraints enable incorporation of more prior knowledge into the fitting. Recently, the TARQUIN algorithm was also developed to automatically analyze high-resolution NMR FIDs using a simulated basis set composed of sub-groups of magnetically equivalent spins (24). In this method, a fixed time domain truncation is used to remove the lipids and macromolecules and, global and basis element specific frequency offsets are determined by evaluating each possibility within a set range. The fixed truncation decreases the SNR of the signals analyzed during the optimization procedure and likely limits the estimation of short T_2 metabolites. Whereas, HR-QUEST avoids the SNR degradation by using a more elaborate approach to model the background. Additionally, HR-QUEST empowers the user to choose the metabolite sub-components that require independent fitting of their δ_s s and T_2^* s, which reduces the number of estimated parameters.

The accuracy and reliability of HR-QUEST's background modeling and metabolite estimation were improved by increasing the number of iterations to 6 and parameterizing the longer T_2^* macromolecules. These enhancements reduced the RMSE to <5% for 79% of the metabolites in the Monte Carlo study and incorporated the parameterized macromolecules into the eCRB calculations. Additionally, they improved the estimation of the shorter T_2^* metabolites, such as the polyamines, along with alanine and glutamate in the measured HR-MAS data. Furthermore, the additional iterations reduced the residual and the partial parameterization affected the portioning of the signal between the metabolite components and the background components. Each parameterized peak has been observed previously in high-resolution mammalian brain spectra and is typically attributed to bound amino acids or mobile lipids (7,25,26,37,38). In several of these studies, the parameterized macromolecules were modeled using inversion recovery data. However, for prostate tissue, the large concentrations of citrate and polyamines void this technique because they have shorter T_1 's (4) and overlap with several of the parameterized peaks. Furthermore, since the samples are slowly degrading during the HR-MAS experiment, the extra inversion recovery experiment causes additional degradation (4,39). While the parameterized peaks are present in many different tissues and diseases, their relative intensities are different. Additionally, their lineshape is likely a function of spin rate because the chemical shift anisotropy will be reduced at higher spin rates.

The reliability achieved in the final fits was also assessed using estimated Cramér-Rao Bounds, which yielded eCRBs <5% for 82% of the metabolites. The remaining metabolites had higher eCRBs because their concentrations were very low in the prostate samples studied. HR-QUEST's quantification capabilities will depend on the signal to noise ratio (SNR) available for a given metabolite and on the overlap between the metabolite of interest

and other metabolites, both of which influence the eCRBs. The SNR is a function of the concentration as well as the number of equivalent protons, the splitting pattern of the metabolite, and the number of scans used to acquire the HR-MAS data. Thus, some metabolites can be quantified at relatively low concentrations because the other factors boost their SNR (e.g. Choline with 9 equivalent protons on its singlet was quantified at concentrations as low as 0.28mM in our data). Whereas other metabolites, such as phosphoethanolamine, require a higher concentration to be correctly quantified. For reliable quantification, the phosphoethanolamine concentration needs to be higher than the choline concentration because it has a multiplet pattern that overlaps with several metabolites and macromolecules. In our data, the phosphoethanolamine concentration had to be higher than 1 mM to be reliably quantified. The eCRB calculation employed in this study also attempted to incorporate fitting errors by using a non-standard noise estimate in the computation. Without this adaptation, the eCRBs would have been lower. Unfortunately, the eCRBs provide a necessary but not sufficient criterion for assessing the reliability and also neglect the interaction between the estimated basis elements and the broad background estimated by the HLSVD. Thus, the reliability was also assessed by evaluating the reproducibility of the background across all the measured spectra. However, this approach was limited by the small biological differences that existed between samples. As a result, some of the variability present in the normalized background plots in Figure 6 was inherent to the samples.

Previously, QUEST was used to quantify HR-MAS data following a decimation with an ER-Filter (Extraction-Reduction of the spectral bandwidth), which reduced the number of data points (23,40). While the ER Filter significantly reduces the computation time for the fitting because of the reduced number of points, it requires that the magnitude of the spectrum be close to zero at the edges of the filter bandwidth to avoid distortions. Consequently, the ER filter did not work well in this study because the data had a larger background signal and required a larger amount of decimation. With HR-QUEST, it was possible to quantify HR-MAS data without decimation. The overall computation time was reduced by processing multiple samples in parallel using a grid computing environment.

Despite all the improvements, HR-QUEST still has some limitations. First, the Lorentzian extra damping was unable to correct for more complex lineshape distortions caused by B_0 inhomogeneities. A measured basis set was used in this study to partially correct for magnet specific distortions. In addition, every attempt was made to produce spectra with symmetric lineshapes and to ensure the linewidth at a peak's base was not broader than a Lorentzian line by optimizing the B_0 homogeneity on both the basis set samples and the tissue samples. However, some residual signal still remained for the more concentrated metabolites, such as lactate. Second, HR-QUEST does not correct for sample specific J-coupling variations that may occur in strongly coupled spins systems like citrate (35,36). Both of these limitations provide opportunities for future improvements to HR-QUEST. Lastly, the polyamine basis FIDs used in this study were not perfectly matched to those present in the prostate tissue samples likely because of their extreme sensitivity to the microenvironment (14).

CONCLUSION

HR-QUEST offers a new option for accurately and reliably quantifying the metabolites present in high-resolution ultra-short T_E spectra from prostate tissue samples with relatively short T_2 metabolites and a significant background component. The semi-automated approach can account for spin system dependent and sample dependent changes in chemical shift and T_2^* and will likely be useful for quantifying high-resolution FIDs from other tissue types as well.

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Abbreviations used

δ_s	Chemical Shift
eCRBs	Estimated Cramér Rao Bounds
ERETIC	Electronic REference To access In vivo Concentrations
HLSVD	Hankel Lanczos Singular Value Decomposition
HR-MAS	High Resolution –Magic Angle Spinning
RMS	Root Mean Square
RMSE	Root Mean Square Error
SD	Standard Deviation
SV	Starting Value
Ala	Alanine
Cho	choline
Cit	citrate
Cre	creatine
Eth	ethanolamine
Gln	glutamine
Glu	glutamate
Gly	glycine
GPC	glycerophosphocholine
GSH	glutathione
Lac	lactate
mI	myoinositol
PC	phosphocholine
PE	phosphoethanolamine
sInositol	sylo- inositol
Spd	spermidine
Spm	spermine
Tau	taurine

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Appendix

This appendix describes additional commands and output incorporated into HR-QUEST. It also includes a more detailed description of the Monte Carlo simulation design and analysis.

Controlling the HLSVD modeling

A new command allows the user to set the minimum linewidth for every component modeled by the HLSVD. With this feature, the user can prevent the HLSVD from modeling any of the metabolites present in the data. Also, the Hankel matrix in the HLSVD was converted from a square matrix to a rectangular matrix. This adjustment improved the reproducibility of the background modeling across different computer operating systems and reduced the likelihood that the modeling would lead to an improper solution (a result with a non-decaying component). If the HLSVD modeling happens to yield an improper solution, HR-QUEST reports a warning. Additionally, a new feature was added to HR-QUEST that allows the user to specify the dimensions of the Hankel matrix, along with the number of components modeled by the HLSVD. Using these commands, the HLSVD modeled 21 components with a minimum linewidth of 9.5 Hz in this study. Furthermore, the Hankel matrix contained 1024 points, 100 rows, and 924 columns.

Output files

In the main HR-QUEST output file, the estimates for the metabolite amount, frequency shift, extra damping, phase adjustment, and eCRB are listed for each metabolite or sub-component. HR-QUEST produces a second output file that contains estimated correlations between the amplitude parameters (41) for all the basis elements. This information allows the user to determine the eCRB for the sum of multiple basis elements.

Monte carlo simulation design and data analysis—The background signal used in the Monte Carlo simulations was created by randomly choosing the amplitude, extra damping, and frequency offset for the macromolecule peaks shown in Figure 2, from a uniform distribution and randomly varying the remaining background components using a Voigt apodization.

The accuracy of the background modeling and metabolite fits for the simulations were assessed by computing the Root Mean Square Error (RMSE) in the time domain between the fitted data and the simulated metabolite data for each simulation. A similar RMSE was computed between the modeled and simulated background data. Next, for all the simulations within one of the 6 fitting scenarios evaluated, the mean and standard deviation (SD) of the metabolite and background RMSEs were computed. The analogous mean and SD were also computed for the Root Mean Square (RMS) of the residual. In addition, the resulting metabolite amounts were compared to the simulated amounts by computing the RMSE for each metabolite for each scenario and expressed as a percentage of the metabolite amounts.

Figure 1.

Flowchart of HR-QUEST Algorithm. The algorithm exploits the fact that the majority of the signal from the macromolecules is contained in the beginning of the FID because the macromolecules have short T_2 's. More specifically, HR-QUEST initially fits the data by using a metabolite basis set and applying a NLLS minimization to the later portion of FID. Then it subtracts the estimated metabolite signal from the measured FID and models the macromolecule signal with an HLSVD. HR-QUEST optimizes the fitting of the metabolites and modeling of the macromolecules by iteratively reducing the number of points excluded from the fitting.

Figure 2.

Plot of the eight parameterized macromolecule peaks that were added to the basis set. The plot contains a representative prostate surgical spectrum (green), the estimated background signal for this sample (dashed, dark blue line), the fitted version of the parameterized macromolecule peaks at 1.3, 1.4, 1.55, 1.7, 2.05, 2.3, 3.0, and 3.3 ppm (light blue), and the component of the background signal modeled with HLSVD (dotted, light blue line). The parameterized macromolecule basis spectra were calculated from an independent set of HR-MAS prostate data by using HLSVD to model the background estimated by HR-QUEST in these samples. The parameterized peaks modeled most of the narrower background peaks, accounting for the longer T_2 components in the background. *Ala*, Alanine; *Cho*, choline; *Cit*, citrate; *Cre*, creatine; *Eth*, ethanolamine; *Gln*, glutamine; *Glu*, glutamate; *Gly*, glycine; *GPC*, glycerophosphocholine; *GSH*, glutathione; *Lac*, lactate; *mI*, myo-inositol; *PC*, phosphocholine; *PE*, phosphoethanolamine; *sInositol*, syllo-inositol; *Spd*, spermidine; *Spm*, spermine; *Tau*, taurine.

Figure 3.

(A) Comparison of the root mean square (RMS) of the residual in the time domain when splitting some of the basis-set metabolite signals (in pink) with the original whole metabolite basis set (in dark blue) for 11 prostate tissue spectra. While the RMS of the residual was reduced from $3.48 \times 10^4 \pm 1.67 \times 10^4$ to $1.65 \times 10^4 \pm 4.60 \times 10^3$ by splitting some of the basis spectra, the improvement was more significant for the normal prostate spectra (Samples 1 to 8) than for the therapy spectra (Samples 9 to 11). (B & C) A representative fit from Sample 3 is shown without and with the split basis set. The green and red lines represent the measured spectra and the estimated or fitted spectrum determined by HR-QUEST, respectively. The background estimation determined by HR-QUEST and the residual are drawn in blue and black, respectively. Splitting a portion of the basis set dramatically improved the fitting. However, the background estimate is still incorrect in some regions and some signal still remains in the residual.

Figure 4.

Plots of the Root Mean Square Error (RMSE) obtained from Monte Carlo simulations of the HR-QUEST fits on HR-MAS data from prostate tissue. The Monte Carlo simulations were based on the signal levels measured in one of the representative samples and consisted of 150 variations in the macromolecules and/or noise present in the representative sample. (A) In general, the overall RMSE for the estimated background and the estimated metabolites was lower with additional iterations and when partially parameterizing (2HLSVD + Fit.) the background signal. (B) Furthermore, the RMSE of the individual metabolite concentrations was typically lower with additional iterations but, the optimal number of iterations varied with metabolite (metabolites are plotted with the largest reduction in RMSE at the left and the smallest reduction at the right). The RMSE was reduced by more than 4% when comparing the 2 and 6 iteration results for all metabolites highlighted with the white background. (C) The metabolite specific RMSE was further reduced for almost all the metabolites by partially parameterizing the background signal. *Note: The RMSE of the individual metabolite concentrations is plotted as a percentage of the actual metabolite concentrations. The RMSE for glutamine (Gln) and glucose (Glc) were plotted using vertical axis on the right while the other metabolites were plotted using the vertical axis on the left. *It.*, Iterations; *Fit.*, Fitting; *HLSVD*, Hankel Lanczos Singular Value Decomposition.

Figure 5.

Representative HR-QUEST fits of HR-MAS spectra from (A) a normal prostate tissue sample and (B) a post-therapy prostate tissue sample. The measured HR-MAS spectra are displayed in green. The fitted spectra, background estimates, and residuals obtained when using 6 iterations and estimating the background with a combination of parametric fitting and HLSVD are displayed in red, long-dashed blue, and grey, respectively. The background estimates and residual obtained when the fitting iterated twice and the background was modeled with HLSVD alone are displayed in short-dashed black and solid black, respectively. The partial parameterization of the background was achieved by adding the peaks shown in Figure 2 to the fitting. After increase the number of iterations and adding the parameterized macromolecule peaks, the background estimates were more realistic, and the residuals were smaller in the highlighted regions. Of particular importance, the large PC present in the therapy samples was more accurately fit with the parameterized macromolecule peaks, which eliminated the larger residual peak highlighted by the arrow at 3.23 ppm in panel B.

Figure 6.

Average (Black) and individual (gray) background estimates for eleven HR-MAS datasets from prostate surgical samples after normalizing for sample-to-sample differences (A & B). The background was estimated by HR-QUEST using an HLSVD alone (A) and with a combination of an HLSVD and the 8 parameterized macromolecule peaks shown in Figure 2(B). For each approach, the data was fit with 2 and 6 iterations as indicated. The average of the absolute value of the residual for all eleven surgical samples was also computed for each scenario (C). The fitting with the partially parameterized macromolecules and 6 iterations had the smallest overall residual, especially in the regions highlighted in gray, and still exhibited a reasonably reproducible background estimate. The arrows highlight the residual caused by poor fitting of the large PC and GPC peaks in the 3 therapy samples included in the average.

Figure 7.

Estimated Cramer Rao Bounds (eCRBs) for the metabolite concentrations from the fits for 11 prostate tissue samples. The numbers adjacent to each bar indicate how many of the prostate tissue samples contained that particular metabolite. The eCRBs are reported as a percentage of the metabolites' concentration. The metabolite fits with concentration eCRBs less than 5% were considered to be reliable. Those between 5 and 10% were considered to be marginally reliable and are highlighted with the light grey background. The metabolites with eCRBs greater than 10% were considered to be unreliable and are highlighted with a dark grey background.

Table 1

List of the metabolites quantified with HR-QUEST along with a description of how some of the metabolites were divided into sub-components for the basis set. Some of the fitting results, such as the chemical shift and concentration, are also included. The metabolite concentrations (Conc.) were computed using only concentrations from the healthy prostate tissue samples with eCRBs $\leq 5\%$. GPC, glycerophosphocholine; PC, phosphocholine; PE, phosphoethanolamine

Metabolite Name	Spectral Sub-Component	Spin System	A_{ref} & A_{mf} Constraints	Solution Chemical Shift	Tissue Chemical Shift	Δf_{mi} (Hz)	Range of Δf_{mi} Difference (Hz)	Conc. (mM)
Alanine	Single	$^3\text{CH}_3$	N/A	1.481	1.481	0.2	N/A	1.0
Choline	Single	$\text{N}(\text{CH}_3)_3$	N/A	3.214	3.212	-1.0	N/A	0.40
Citrate	X	CH_A	None	2.565	2.547	-9.1	X-Y: 3.00	8.8
	Y	CH_B		2.695	2.711	7.8		
Creatine	Single	$\text{N}(\text{CH}_3)$	N/A	3.034	Reference	Ref.	N/A	1.4
Ethanolamine	X	$^2\text{CH}_2$	α :X-Y	3.13	NR	NR	NR	NR
	Y	$^1\text{CH}_2$		3.82	NR	NR		
	X	$^4\text{CH}_2$	α :X-Y	2.35	2.352	1.1	X-Y: 0.89	2.3
		$^3\text{CH}_2$		2.1	2.103			
Glutamine	Y	^2CH		3.7652	3.768	1.3		
	X	$^4\text{CH}_2$	α :X-Y	2.45	NR	NR	NR	NR
		$^3\text{CH}_2$		2.14	NR			
	Y	^2CH		3.78	NR	NR		
Glutathione	X	$^4\text{CH}_2$	None	2.555	2.547	-3.8	X-Y: 14.96	10.78
	Y	^2CH & $^{10}\text{CH}_2$		3.785	3.785	0.1		
		$^3\text{CH}_2$		2.167	2.167			
		^7CH		4.584	4.584			
Glycine	Single	$^7\text{CH}_2$		2.965	2.965			
		$^2\text{CH}_2$	N/A	3.561	3.563	1.0	N/A	1.2
	Single	$\text{N}(\text{CH}_3)_3$	α : GPC - PC f: GPC - PC	3.238	3.232	-3.0	N/A	0.41
	X	$^3\text{CH}_3$	None	1.335	1.338	1.3	X-Y: 2.06	13
Lactate	Y	^2CH		4.13	4.135	2.6		

Metabolite Name	Spectral Sub-Component	Spin System	A_{inf} & A'_{inf} Constraints	Solution Chemical Shift	Tissue Chemical Shift	Δf_{mi} (Hz)	Range of Δf_{mi} Difference (Hz)	Conc. (mM)
myo-Inositol	W	⁵ CH	α : W-Y-Z	3.28	3.274	-2.8	Z-W,X,Y: 0.38	6.4
	X	¹ CH& ³ CH	f: W-X-Y	3.54	3.534	-2.8		
	Y	⁴ CH & ⁶ CH		3.62	3.614	-2.8		
	Z	² CH		4.068	4.061	-3.7		
PC	Single	N(CH ₃) ₃	α : GPC-PC f:GPC-PC	3.230	3.224	-2.9	N/A	0.22
PE	X	² CH ₂	α :X-Y	3.217	3.236*	9.3	X-Y: 3.05*	1.3
Spermidine	Y	¹ CH ₂		3.976	3.973	-1.0		
	W		α : W-X-Y-Z	1.765	1.784	9.3	W-X: 5.09 W-Y: 19.11 W-Z: 3.65	3.8
	X			2.075	2.102	13.7	X-Y: 15.70 X-Z: 5.96	
Spermine	Y			3.05	3.052	0.9	Y-Z: 16.33	
	Z			3.115	3.108	-3.5		
	X		α : X-Y-Z	1.765	1.759	-3.2	X-Y: 5.38 X-Z: 2.57	
	Y			2.065	2.091	12.8	Y-Z: 3.10	
Syllo-Inositol	Single	¹⁻⁶ CH	N/A	3.348	3.349	0.6	N/A	0.58
Taurine	Single	¹ CH ₂	N/A	3.421	3.419	-1.0	N/A	2.9

[†] These values were somewhat uncertain because these metabolites have a lower SNR and/or significantly overlapped with other metabolites.

^{††} The concentration reported for spermidine is the sum of spermidine and spermine.

NR: Not Reported – The eCRB's for glutamine and ethanolamine were > 5% for the majority of the samples. Consequently, the fitted results were not reported for these metabolites.