# QUESP and QUEST revisited – fast and accurate quantitative CEST experiments

## Part I: single far off-resonant pool, cw saturation

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Word count: 4980

**List of abbreviations**

| | |
|---|---|
| BM | Bloch-McConnell |
| CEST | chemical exchange saturation transfer |
| DOTA | 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate |
| MT | magnetization transfer |
| $R_{ex}$ | exchange-dependent relaxation |
| $R_{1\rho}$ | longitudinal relaxation in the rotating frame |
| SNR | Signal-to-noise ratio |
| QUESP | quantification of exchange rate as a function of saturation power |
| QUEST | quantification of exchange rate as a function of saturation time |

# Abstract

Purpose: Chemical exchange saturation transfer (CEST) NMR or MRI experiments allow detection of low concentrated molecules with enhanced sensitivity via their proton exchange with the abundant water pool. Be it endogenous metabolites or exogenous contrast agents, an exact quantification of the actual exchange rate is required to design optimal pulse sequences and/or specific sensitive agents.

Methods: Refined analytical expressions allow deeper insight and improvement of accuracy for common quantification techniques. The accuracy of standard quantification methodologies, such as quantification of exchange rate using varying saturation power (QUESP) or varying saturation time (QUEST), is improved especially for the case of non-equilibrium initial conditions and weak labeling conditions, meaning the saturation amplitude is smaller than the exchange rate ($\gamma B_1 << k$).

Results: The improved analytical QUESP/QUEST equations allow for more accurate exchange rate determination, also providing clear insights on the general principles to execute the experiments and to perform numerical evaluation. The proposed methodology was evaluated on the large-shift regime of paramagnetic CEST (paraCEST) agents using simulated data and data of the paramagnetic Eu(III) complex of DOTA-tetraglycineamide.

Discussion & Conclusion: The refined formulae yield improved exchange rate estimation. General convergence intervals of the methods which would apply for smaller shift agents are also discussed.

# Introduction

$^1$H CEST is an MRI technique capable of indirectly detecting millimolar concentrations of exchangeable protons with molar sensitivity via the water signal. Exchangeable protons of endogenous or exogenous compounds resonate at distinct frequencies relative to the water protons and can therefore be selectively saturated by RF irradiation. In addition, given the low solute concentration (µM-mM range) compared to water protons (approximately 111 M), prolonged saturation periods provide an amplification process for the small solute signal which produces a reduction in water signal. This capability for amplified detection has allowed the design of new families of contrast agents such as paramagnetic CEST agents enabling, for instance, measurement of pH, temperature and buffer condition (1–7), or diamagnetic CEST agents such as the thymidine analogs(8), imidazoles, salicylates (9–11) and iodinated agents (12–14). These probes contain protons that resonate at multiple frequencies, thus enabling a multi-color imaging, similarly to optical imaging agents (15–17).

Spectroscopy based methods have been proposed for measuring exchange rates of slowly exchanging systems (18,19), however, they are not suitable for fast exchange rate quantification due to severe line broadening. On contrary, it is possible to provide a quantification for experiments in this regime with CEST and data can be interpreted by the Bloch-McConnell (BM) differential equations that incorporate parameters such as free precession, excitation and relaxation, as well as the exchange between different spin pools.(20)

McMahon et al were the first to develop an MRI compatible method to measure exchange rate based on exploiting the influence of saturation time and saturation power on signal intensity(21). Using a simplified solution of the BM equations as described in the work of Zhou et al. (22,23), McMahon and colleagues calculated exchange rates by changing either the labeling efficiency (by the saturation pulse power) or the saturation time (21), and compared their results with the fitting of the BM differential equations in time, which forms a gold standard for quantification of CEST (24).

The existing analytical solutions that describe the above mentioned experiments are well suited for calculation of exchange rates, as long as a strong labeling is achieved. ‘strong labeling’ means in this context that the saturation amplitude is larger than the exchange rate ($\gamma B_1 > k$), thus the magnetization of the CEST pool is close to 0 during irradiation and the CEST effect is maximized as well as the labeling efficiency α, which approaches 1.

In this work we show that weak labeling ($\gamma B_1 < k$, $\alpha < 0.5$), as well as non-equilibrium initial magnetization conditions may result in inaccurate quantification of exchange rates if the existing analytical solutions are used. We have therefore focused on this issue and derived analytical QUESP/QUEST formulae based on the equivalence of the spin-lock theory to CEST experiments (25,26). The derived equations were applied on a system with large chemical shifts where direct water saturation is negligible. We show that these formulae agree with the original QUESP/QUEST equations for strong labeling, furthermore they extend the convergence interval for weak labeling. In addition to QUESP, we apply the similar Ω-plot method, which reformulates QUESP to a linear regression problem (27), using the same data. For the experimental validation of our formulae, we used paramagnetic CEST (paraCEST) agents (28) due to the following advantages. First of all, these agents induce large shifts for exchanging protons, simplifying the theory (direct saturation does not have to be taken into account). Secondly, the knowledge of the exact exchange rate is very important for the design of novel and potentially useful classes of paraCEST molecules and they are therefore the ideal test molecules for this application (29,30).

In this work, we show that data interpretation can be incorrect already for this simple case, thus indicating the importance of extending the quantification methodology at this level. Moving to more complex cases and CEST effects closer to bulk water frequency is even more challenging, and these aspects will be analyzed in a forthcoming study. Moreover, the aim of this paper is to provide researchers interested in quantitative CEST experiments with a source code freely available for deducing exchanging rates and for reproducing the results of this article.

# Theory

CEST results are usually presented in so-called Z-spectra. To obtain a Z-spectrum, the amplitude of the saturated water magnetization ($M_{sat}(\Delta\omega)$), normalized as a proportion of the unsaturated water magnetization ($M_0$), is plotted as a function of the saturation pulse frequency $\omega$. As the acquired signal $S_{sat}$ or $S_0$ is proportional to the magnetization, the normalized Z-spectrum is described by the value $Z(\Delta\omega)$ given as equation [1] (31):

$$Z(\Delta\omega) = S_{sat}(\Delta\omega)/S_0 = M_{sat}(\Delta\omega)/M_0 \qquad [1]$$

A typical pulse sequence diagram that is being used for quantitative CEST experiments and the principle behavior in time of the Z-magnetization is presented in Figures 1a and 1b, respectively. The depicted diagram shows the preparation at one frequency offset, with subsequent readout of the water signal either by an FID or a fast imaging readout. Assuming a dummy scan, the magnetization $M_{pre}$ recovers during $t_{rec}$ time with relaxation defined by the water relaxation time $T_{1a}$ to the value $M_{i,}$ which is the initial magnetization right before the saturation module (i.e $M_i$ $\neq M_0$). During the saturation pulse of duration $t_p$, the magnetization follows an $R_{1p}$ decay, reaching the z-magnetization with the value $M_{sat}$ right after irradiation. For long enough saturation conditions, (e.g. $t_p >> T_{1a}$) $M_{sat}$ approaches the saturation steady-state. During the acquisition module, the z-magnetization decreases to $M_{post}$, e.g., a short 90° pulse would set it to zero, or a gradient-echo readout would approach an imaging steady-state. Finally, after the readout the magnetization is $M_{post}$. The CEST experiment for a single offset is now complete. However, to acquire a full Z-spectrum the saturation frequency is now changed and the sequence is played out again with the condition $M_{pre}=M_{post}$ (assuming that $M_{post}$ does not depend on $M_{sat}$).

In many cases instead of using $M_0$, the data is often normalized employing magnetization after far off-resonance irradiation. This can lead into problems especially if the sum of saturation time and recovery time is shorter than $T_{1a}$ (Figure 1c). Then $M_{offres}<M_0$ and all normalized effects are artificially increased. In such case, as shown in detail in the Supporting Information (Figure S1-S3), the quantification equations are not directly applicable. However, even if an additional $M_0$ signal is measured after long relaxation in a separate scan, the timing of the CEST sequence can still lead to an initial magnetization before saturation $M_i<M_0$, that can influence the saturated signal. Only after a long recovery time before each saturation ($t_{rec}$ > 5xT1a) all previous magnetization history is lost regardless the sequence timing (so $M_i=M_0=M_{offres}$). Generally, both $M_0$ and $M_i$ must be known to be able to interpret quantitative CEST experiments for arbitrary initial and saturation conditions. If $t_p$ is short compared to $T_{1a}$, the measured value will depend on the initial value $M_i$. If $t_p$ is long compared to $T_{1a}$, the magnetization approaches the saturation steady-state.

To evaluate the CEST signal, two Z-values are commonly used: the label scan, which is the normalized z-magnetization after saturation at the CEST resonance, $Z_{lab} = Z(\Delta\omega = +\delta\omega_b)$, and the reference scan which represents the saturation without CEST effect and it can be estimated from the baseline or, as here, from the opposite frequency, $Z_{ref} = Z(\Delta\omega = -\delta\omega_b)$.

The CEST effect can then be defined by the magnetization transfer ratio asymmetry ($MTR_{asym}$, equation [2]) or the inverse asymmetry of the normalized z-magnetization ($MTR_{Rex}$, equation [3]) as the difference in signal on either side of the water peak centered at 0 ppm for constant amplitude $B_1$.

$$MTR_{asym} = Z_{ref}(B_1) - Z_{lab}(B_1) = \frac{M_{sat}(-\delta\omega_b)}{M_0} - \frac{M_{sat}(+\delta\omega_b)}{M_0} \qquad [2]$$

$$MTR_{Rex} = \frac{1}{Z_{lab}(B_1)} - \frac{1}{Z_{ref}(B_1)} = \frac{M_0}{M_{sat}(+\delta\omega_b)} - \frac{M_0}{M_{sat}(-\delta\omega_b)} \qquad [3]$$

Detailed theoretical descriptions and derivations of the Z-values and $MTR_{asym}$ are given in appendix A. In this section we only provide the final formulae we used for data evaluation. Also, the initial Z–magnetization before saturation is written as $Z_i = M_i/M_0$ in the following sections.

## 1. Analytical solution for equilibrium magnetization $M_0$ as the initial magnetization $M_i$

We consider a two pool system of the water pool (pool a) with thermal magnetization $M_{0a}$ and the CEST pool (pool b) with thermal magnetization $M_{0b}$ and the relative fraction $f_{b=}$ $M_{0b}/M_{0a}$. We assume that the initial magnetization at thermal equilibrium is $M_i = M_0$, thus $Z_i$=1. It can be shown that then and only then the CEST effect can be described quantitatively by the equation [4], or in other words – only then equation [24 (Appendix A) simplifies to equation [4].

$$MTR_{asym}(\alpha(B_1), t_p) = \frac{R_{ex}^{lab}}{R_{1a} + R_{ex}^{lab}}\left(1 - e^{-(R_1 + R_{ex}^{lab})t_p}\right) = \frac{f_b k_b \cdot \alpha}{R_{1a} + f_b k_b \cdot \alpha}\left(1 - e^{-(R_{1a} + f_b k_b \cdot \alpha)t_p}\right) \qquad [4]$$

For the so called labeling efficiency α, different limits are published (31), we assume here large shifts between water and the CEST pool, then α reads as in the equation [5], where $k_b$ is the exchange rate, $R_{2b}$ is the transversal relaxation rate of the CEST pool and the $\omega_1 = \gamma B_1$ is the rf saturation amplitude.

$$\alpha(B_1) = \frac{\omega_1^2}{\omega_1^2 + k_b(k_b + R_{2b})} \qquad [5]$$

In the original QUEST/QUESP paper it was assumed that a steady-state in pool b is reached instantaneously upon saturation of the solute and that the exchangeable proton pool is completely isolated, meaning that $M_b$=0. The authors introduced an $MTR_{asym}$ given by the equation [6]:

$$MTR_{asym} = \frac{f_b k_b \cdot \alpha}{R_{1a} + f_b k_b}\left(1 - e^{-(R_{1a} + f_b k_b)t_p}\right) \qquad [6]$$

Comparing equation [6 to equation [4, the two $\alpha$ factors appear additionally that scale the product of the fractional concentration and the exchange rate $f_b k_b$. The additional appearance of $\alpha$ can be understood conceptually as instantaneous saturation of pool b to the value $M_b = (1-\alpha) \times M_{b0}$; this means $M_b \approx 0$ for strong labeling and $M_b = M_{b0}$ for labeling approaching zero (see reference (32) for more details).

In this manuscript we show that by using equation [4 and including the $\alpha$ terms, improved estimates of the exchange rates can be calculated. Note also that equation [6 becomes equal to equation [4 for strong labeling, thus when $\alpha = \frac{\omega_1^2}{\omega_1^2 + k_b^2} \approx 1$, but also when $R_{1a} >> k_b f_b$. We want to point out that the original definition of α (in ref. (23), $\alpha = \omega_1^2 / (\omega_1^2 + pq)$ ) is not valid in the case of the exchange rates of paraCEST agents ($k_b > R_{1a}/f_b$, for more details see Supporting Information).

Using equations [4[ or [6], two experiments can be designed to quantify exchange rates from the water. A QUESP experiment can then be understood as acquisition of $MTR_{asym}(B_1)$ for varying $B_1$ at a fixed saturation duration $t_p$ , whereas a QUEST experiment can be understood as $MTR_{asym}(t_p)$ for varying $t_p$ at a fixed saturation amplitude $B_1$.

Equation [6] can be used for arbitrary saturation times $t_p$, however, for the sake of completeness we provide the original and revised QUESP equations [7] and [8], respectively, in steady state (i.e $t_p \rightarrow \infty$)

$$MTR_{asym} = \frac{f_b k_b \cdot \frac{\omega_1^2}{\omega_1^2 + k_b^2}}{R_{1a} + f_b k_b} \qquad [7]$$

$$MTR_{asym} = \frac{f_b k_b \cdot \frac{\omega_1^2}{\omega_1^2 + k_b^2}}{R_{1a} + f_b k_b \cdot \frac{\omega_1^2}{\omega_1^2 + k_b^2}} \qquad [8]$$

The theory described in this section can be used in two ways to yield correct estimates of exchange rates. Firstly, if the recovery time $t_{rec}$ (i.e. the delay time before the saturation, see Figure 1) is long enough so that the initial magnetization is fully relaxed (i.e. $Z_i=1$), then arbitrary saturation times $t_p$ can be used and fitted by equation [4. Secondly, if the saturation time $t_p$ is long enough (>3×$T_1$) so that the saturation steady-state is reached, and therefore is independent of $Z_i$, then exchange rate quantification via QUESP experiments is possible by using equations [7 and [8.

### Omega plot methods for steady-state QUESP

In saturation steady-state, employing the inverse asymmetry $MTR_{Rex}$, exchange rates can additionally be calculated using the equation [9], which eliminates spillover and semi-solid MT and relates $MTR_{Rex}$ to $k_b$, $\omega_1$, $f_b$ and $R_{1a}$.

$$MTR_{Rex} = \frac{1}{Z_{lab}(B_1)} - \frac{1}{Z_{ref}(B_1)} = \frac{1}{R_{1a}} f_b k_b \cdot \frac{\omega_1^2}{\omega_1^2 + k_b^2} \qquad [9]$$

In addition, using the $1/MTR_{Rex}$, the exchange rate can be obtained from the X-intercept of a plot of steady-state CEST intensity as a function of $1/\omega_1^2$. The derived equation [10] was given by Meissner et al (33).

$$y\left(\frac{1}{\omega_1^2}\right)=\frac{1}{\frac{1}{Z_{lab}}-\frac{1}{Z_{ref}}}=\frac{\mathrm{R}_{1\mathrm{a}}}{f_b k_b}+\frac{\mathrm{R}_{1\mathrm{a}} k_b}{f_b}\cdot\frac{1}{\omega_1^2} \qquad [10]$$

Equation [10 is referred as the Ω-plot method and has been originally introduced by Dixon et al. (27). The original Ω-plot formula is given by the equation [11 which is a special case of equation [10 for $Z_{ref}=1$.

$$y\left(\frac{1}{\omega_1^2}\right)=\frac{Z_{lab}}{1-Z_{lab}}=\frac{1}{\frac{1}{Z_{lab}}-1}=\frac{\mathrm{R}_{1\mathrm{a}}}{f_b k_b}+\frac{\mathrm{R}_{1\mathrm{a}} k_b}{f_b}\cdot\frac{1}{\omega_1^2} \qquad [11]$$

## 2. Analytical solution for arbitrary initial magnetization $M_i$

The theory as described above can be extended to account for non-thermal equilibrium initial magnetization $Z_i$ by using equation [24 (Appendix A); with $R_{ex}$ (equation [21, (Appendix A)) inserted, it results in equation [12] that shows an explicit dependency on the initial magnetization before the saturation module $Z_i=M_i/M_0$ (see Figure 1b).

$$MTR_{asym}=Z_{ref}(t_p,B_1)-Z_{lab}(t_p,B_1)=\frac{f_b k_b\cdot\alpha}{R_{1a}+f_b k_b\cdot\alpha}+(Z_i-1)e^{-R_{1a}t_p}-\left(Z_i-\frac{R_{1a}}{R_{1a}+f_b k_b\cdot\alpha}\right)e^{-(R_{1a}+f_b k_b\cdot\alpha)t_p} \qquad [12]$$

Therefore, for fast and accurate quantitative experiments, we suggest the following:

1. Measuring of $M_0$ for a recovery time $t_{rec}$ equal to 5×$T_1$ and then use this $M_0$ for normalization of all Z-spectra. Alternatively, $M_{far\text{-}offres}$ for very long saturation time (5×$T_1$) can be used as $M_0$ estimation;
2. Measuring the initial magnetization right before saturation $Z_i=M_i/M_0$. This can be achieved by running the sequence with the same timing, but removed saturation block)
3. Measuring Z-spectra
    a. For fully relaxed initial magnetization
    b. Speed-up fashion: decreased recovery and saturation times for QUEST and QUESP as long as SNR is sufficient
4. Measuring $T_1$ of the sample;
5. Fitting the data by the full BM equations or using equation [12 and $R_1$, $M_i$ and $M_0$.
   (as described in the Supporting information, the normalization of the data and the fit must be the same)

# Methods

### Simulation and full Bloch-McConnell fitting

Numerical simulations and experimental data were processed using custom-written scripts in Matlab (MATLAB version 8.2.0.701 Natick, Massachusetts: The MathWorks Inc., 2013) following previously reported procedures (24). To evaluate the revised formulae (equations [4,[6,[12), chemical exchange saturation transfer data was simulated using a rectangular pulse shape with pulse duration $t_p$ and amplitude $B_{1.}$ The CEST effect was evaluated using an exchanging system of $k_b$=9000 Hz and 1000 Hz, respectively. The relative proton concentration corresponds to 15 mM agent in the solvent system): $f_b$= 15 mM / 111 M = 0.000135. In addition, the water pool relaxation is chosen similar to typical values of phantoms with $R_{1a}$=0.3 Hz and $R_{2a}$=0.5 Hz, relaxation parameters of the CEST pool were set fixed to $R_{1b}$=1 Hz, $R_{2b}$=50 Hz. The static magnetic field was set to $B_0$=7.0 T. In order to sample the chemical shift of a CEST agent at 50 ppm, Z-spectra were sampled between -80 and 80 ppm. Rician noise of 0.1 % of $M_0$ was added to the simulated Z-values. This was repeated for different saturation powers $B_1$ between 10 µT and 35 µT and saturation times $t_p$ between 0.5 s and 10 s.
For fitting of experimental data the two pool system was extended to a three pool model; the third amide pool resonating upfield from water was added to the employed fitting model according to Dixon et al (27) and (34); the additional amide pool was initialized by the parameters $\delta\omega_{amd}$=-6.0 ppm, $f_{amd}$=0.5 ‰, $k_{amd}$=50 Hz. $R_{2amd}$=50 Hz, and did not influence the PARACEST pool quantification.

Analytical solutions of the BM equations were fitted in Matlab employing the optimization function `lsqcurvefit`. All simulation and evaluation files can be found and downloaded from the websites cest-sources.org or github.org:
**https://github.com/cest-sources/BM_sim_fit/tree/7b7c7ee585a3e93725336af5cd5a627cba14b2ce**

### Preparation of paraCEST contrast agent

The experimental results reported in this work were obtained using EuDOTAM-Gly, a complex of Eu(III) with the tetraglycineamide derivative of DOTA. This complex was prepared according to the previously reported procedure (2). The agent was dissolved in $H_2O$:$D_2O$ (9:1, v/v) at a concentration of 10 mM and pH=7.4.

### CEST experiments

All experiments were performed on a 7T (300.17 MHz) Bruker Avance III NMR spectrometer (Bruker, Ettlingen, Germany) using a rectangular saturation pulse followed by a 90°-FID readout. The recovery time $t_{rec}$ after readout was 3 s. The saturation transfer experiments were carried out at a temperature range 10-50 $^{o}$C by irradiating the sample at increments of 1 ppm with frequency range ±80 ppm. Spectra were measured by recording the bulk water signal intensity as a function of the presaturation frequency. Saturation offsets are reported relative to the signal of bulk water. The used temperatures were corrected by measuring the frequency difference of neat ethylene glycol at each temperature reported (35).

For each temperature, data was collected by varying the saturation power whilst the saturation time remained constant (10 s). The saturation field strengths used were 10, 15, 20, 25, 30 and 35 µT. Longitudinal relaxation times were obtained in an independent experiment using the standard inversion recovery with 1% gradient to eliminate the radiation damping effect. Steady-state CEST experiments were performed using a 5 mm NMR tube, however, for CEST experiments with short saturation ($t_{rec}$=1 s, $t_p$=3 s) the volume was reduced by a factor of 0.1 to avidavoid radiation damping influences by using a smaller 2 mm NMR tube (1.6 mm inner diameter) filled with the sample, inserted into the 5 mm NMR tube filled with $D_2O$.

### Data evaluation

For data evaluation, the CEST effect was calculated employing the asymmetry analysis $MTR_{asym}$ or the inverse asymmetry of the normalized Z-magnetization $MTR_{Rex}$. Since the label and reference values are defined as $Z_{lab} = Z(\Delta\omega = +\delta\omega_b)$ and $Z_{ref} = Z(\Delta\omega = -\delta\omega_b)$, the $MTR_{asym}$ and $MTR_{Rex}$ are calculated using equations [2] and [3].

### QUEST/QUESP validation

For arbitrary saturation but thermal initial conditions (Zi=1), the original QUESP/QUEST equation given by equation [6 was compared to the revised version (equation [4).

For arbitrary initial conditions (Zi≠1) the results obtained using equation [12 were compared to the outcome of equations [4 and [6, respectively. In all simulations and equations negligible direct saturation of the water protons was assumed, as well $R_{2b}$ was assumed to be negligibly small compared to $k_b$ and was set to 0.

### Exchange rate as a function of temperature

The Arrhenius equation [13] allows to determine the exchange rate constant of a first-order exchange process as a function of temperature T(36), where $k_c$ (298.15 K) is the collision frequency factor at 298.15 K, $E = E_A + \Delta H_R^0$ with the activation energy $E_A$, $\Delta H_R{}^0$ is the standard reaction enthalpy for the self-dissociation of water (55.84 $\frac{kJ}{mol}$) (37), and R is the ideal gas constant (8.314$\frac{J}{mol\ K}$).

$$k_b(T) = k_c(298.15\ K)\cdot\frac{mol}{l}\cdot 10^{\frac{E}{R\cdot \ln 10}\left(\frac{1}{298.15\ K}-\frac{1}{T}\right)} \qquad [13]$$

# Results

The principle methods for quantification of exchange rates by varying saturation power or varying saturation time are shown in the Figures 2a,b or 2c,d, respectively. In addition, CEST Z-spectra at constant time and power with varying the exchange rate are shown in Figure 2e,f. The comparison in Figure 2b,d,f of the analytical descriptions of $MTR_{asym}$ (lines) with BM simulation (circles) verifies that the revised equation (equation[4) improves the original equation [6 (dashed lines in Figure 2b,d,f), especially in the case of weak labeling ($\gamma B_1 << k$).

### QUESP and Ω-plot method in steady-state

Simulated Z-spectra data of two different $k_b$ values of 1000 and 9000 Hz, employing saturation powers of $B_1$=5 µT to 35 µT in steps of 5 µT and saturation time $t_p$ = 100 s (steady-state), were used to evaluate the simplified QUESP solutions and the Ω-plot method (Figure 3). The obtained results indicate that the original QUESP formula (equation [6) leads to overestimated $f_b$ and underestimated $k_b$ values (blue lines Figure 3a-b), when compared to the revised QUESP formula (equation [4, red line, Figure 3a-b) which shows an improved estimation, closer to the actual concentration ($f_b$ = 1.35×10$^{-4}$) for both exchange rates. Consequently, the inclusion of the $\alpha$ factor in equation [4 that is much smaller than 1 (i.e. $\alpha$=0.35), is the key parameter which enables accurate estimates of exchange rates.

For steady state conditions, the QUESP approach using inverse asymmetry (Figure 3c-d), as well as the $\Omega$-plot method (Figure 3e-f), yielded the best estimation for $k_b$= 9000 Hz and satisfactory results for $k_b$=1000 Hz. One has to keep in mind that the statistical errors for the $\Omega$-plot method are different for each point in the plot and should be used as weights for the linear fit. Otherwise, the highest data point (which results from the lowest power) has too much influence to the least-squares fit and thus the outcome can be biased. In this work, we assumed constant noise and used MTR (or 1/y) as weights for the least-squares fit.

### QUEST – non steady-state, fully relaxed initial condition

Unlike QUESP, the QUEST analysis using a single offset does not allow for a full quantification of $f_b$, $k_b$, and $R_{2b}$, as no separation of $k_b$ and $f_b$ in $R_{ex}$ (see equations [18 and [21, Appendix A) is possible by QUEST. Consequently, the concentration $f_b$ must be provided to the single offset QUEST fit in order to execute the exchange rate estimations.

### Simplified QUEST solution

Similarly to the QUESP tests (see above), we performed QUEST evaluations for two different $k_b$ values (1000 and 9000 Hz, respectively) for saturation power of $B_1$ = 10 μT, and the saturation times 0.5, 0.75, 1, 2, 3, 4, 5, 7.5, and 10 s. The relative concentration of $f_b$=1.35×10$^{-4}$ has been already provided and fixed (Figure 4). Both solutions using the original and revised QUEST formulae (equations [6 and [4, respectively) provide similar results for the lower exchange rate (Figure 4a). However, the original QUEST formula (equation [6 blue line) does not fit the data for given $f_b$ and underestimates $k_b$ with 3900 Hz for the higher exchange rate of 9000 Hz (Figure 4b). On the other hand, the revised QUEST formula (equation [4) yields an improved estimation with an exchange rate of 9139±87 Hz.

### QUESP and QUEST with arbitrary initial and saturation conditions

Until now the important conditions of a fully relaxed initial magnetization (thus long recovery time) or a steady-state saturation (thus long saturation time) needed to be fulfilled to get accurate parameter estimation. To be able to interpret speed-up experiments, also QUESP and QUEST estimations with arbitrary initial magnetization before saturation were considered. We assumed the same system as before (see Figure 3a-b), yet using an initial magnetization of $M_i$ = 0.3×$M_0$ or $Z_i$ = 0.3 to perform QUESP (Figure 5a-b). Using the revised equation including Zi (equation [12), the estimation and fitting procedure improves significantly, resulting in values close to initially provided ones. It is important to note that different QUESP equations actually fit the data very well. However, the estimated parameters are very different across different equations used. In turn, the plausibility of obtained results is seriously compromised, despite the ‘good’ fits to the given data.

When the QUEST estimations are performed for Zi≠0, the outcome is similar than for QUESP at $M_i$=0.3×$M_0$ or $Z_i$=0.3 (Figure 5c-d). If $M_i$ and $M_0$ are provided, the extended equation [12 yields good estimates. On the other hand, all other equations yield in worse fits and biased estimations for exchange rates.

**Experimental results**

Following the calculations with simulated parameters (see above), we performed a series of multi-$B_1$ experiments with EuDOTAM-Gly between 10 and 30 °C with the saturation having reached a steady-state (Figures 6 and 7). The three pool BM fits show a good match to the Z-spectra and exchange rates are determined to be approx. 3000 Hz at 13.4 °C and around 7000 Hz at 23.8 °C (Figure 6 a,b). When the original QUESP formula was employed, an exchange rate of 2000 Hz and 3200 Hz at 13.4 °C and 23.8 °C, respectively, were obtained. Finally, the improved formula for QUESP and the Ω-plot method yielded much better exchange rate estimations of 2700 Hz and 6000 Hz at 13.4 °C and 23.8 °C, respectively. When the estimations are expanded to four sets of experimental data with temperatures ranging between 10-30 °C and results are compared across the different methods (Figure 7a), the exchange process (evaluated by the Arrhenius parameters, equation [13) can be determined with best accuracy using the BM fit (E= (59.4 ± 10) kJ/mol, $k_c$=(7657 ± 495) Hz), followed by the revised Ω-plot and QUESP methods. The original QUESP equation (equation [6) yields strong underestimations of the Arrhenius exchange parameters E= (30.7 ± 8.6) kJ/mol, $k_c$=(3379 ± 168) Hz (data not shown).

If an even larger temperature range is used (T≈10-50 °C) (Figure 7b), the exchange rates increase up to 50 kHz. This violates the required condition “chemical shift difference > CEST peak width” (31), and thus the regime of validity of the simplified formulae for the labeling efficiency (equations [21 and [19, Appendix A), as discussed below. In such case, only the full BM equation yields reliable results and match well to the Arrhenius equation [13 at all temperatures, followed in accuracy by Ω-plot and finally the revised QUESP method (Figure 7b).

To show the improvement in the regime of short saturation and arbitrary initial conditions, Z-spectra with a recovery time of only 1 s and a saturation time of 3 s were acquired for the temperatures 13.4 °C and 23.8 °C (Figure 8). Because of the short recovery, the initial magnetization before saturation was measured to be $Z_i$=0.865 at 13.4 °C and $Z_i$=0.769 at 23.8 °C. This was provided to the BM fit algorithm along with the measured R1a values of 0.42 Hz and 0.33 Hz, respectively, to allow fitting of the multi-$B_1$-Z-spectra, showing a baseline < 1 (Figure 8ab). The results of this speed-up experiment match well with the steady-state outcome (Figure 6). For the evaluation of the improved theory, we compared the original equation with the revised equation for $Z_i$=1 (equation [4), and the revised equation for arbitrary $Z_i$ (equation [12). For both temperatures, equation [12 shows the best results, when compared to the full BM fit (Figure 8cd). For the lower temperature the improvement is negligible as $Z_i$ is already close to 1 due to the faster $T_1$ relaxation, while the improvement becomes significant for 23.8 °C. Interestingly, we note that the Ω-plot method yields still relatively good exchange-rate estimation, despite being used outside of its expected convergence regime of steady-state saturation (Figure 8ef).

# Discussion

This work is an extension for the quantitative analytical description of QUEST and QUESP methods (21,23,38), improving the case of weak labeling ($\gamma B_1$<k). The above presented results show this is especially important when evaluating faster exchange rates that often result in lower labeling efficiencies. In addition, we extended the formulae for arbitrary initial conditions for the z-magnetization. Before that, the design of a quantitative CEST experiment required a fully relaxed system (i.e. a system that has reached thermal equilibrium magnetization before saturation) or a system which is close to a saturation steady-state in order to simplify the data analysis. However, these experimental settings are time-consuming and the results are biased in the case of non-

equilibrium initial magnetization. In this work we demonstrated that the analysis of data with arbitrary initial condition yields accurate estimates of exchange rates only when the initial magnetization is known and taken into account. This is not only important if analytical evaluation formulae are used, but also if full numerical BM equations are used; the insight of the analytical formulae show that the initial magnetization needs to be known. As shown in the Supporting Information, different normalization can hide this necessity leading to fit matching the data, but wrong estimations. Furthermore, considering the existence of several evaluation methods, we compared the methods that have been widely used in the literature in order to assess which method is the best, i.e. the most convenient, without compromising the accuracy of the obtained results.

To this end, if scanning time is not an issue, the best quantification is clearly achieved with multiple $B_1$-Z-spectra fitting using the BM equation. Alternatively, if the scan time is limited and only certain number of offsets can be acquired, we show here that the revised QUESP and Ω-plot methods yield estimations close to the real value in simulations or the BM fit results in experiments. Compared to fitting single offset QUESP data using the analytical expression, the Ω-plot method additionally cancels spillover effects (33) because it is implicitly based on the spillover-corrected inverse metric of the steady-state Z-spectrum (27,39). Although this is a steady-state method requiring long saturation, its features and the resulting accuracy shown in here suggest Ω-plot method as the method of choice for many quantitative CEST applications. A limitation of this method is the number of pools that can be accurately quantified – if handling two or more pools with similar frequencies, the linearity of the Ω-plot is lost and simple linear regression is not possible anymore (see equation [10). In such case, a linear superposition of QUESP (equation [4) might be more promising. We want to point out that Sun et al. showed the inverse metric approach, with its spillover correction ability, is also applicable in non-steady-state as the transient state of the inverse metric decays relatively quickly (40), this could be the explanation why we still observed good estimation of exchange rates in the speed-up experiment (Figure 8).

In case the scan time needs to be reduced even more, the revised QUESP method for arbitrary conditions (equation [12) allows for reliable interpretation of data. Here, the prerequisite is knowledge of the initial magnetization $M_i$ just before the saturation; then the saturation does not need to reach steady-state and can still be evaluated. Consequently, we demonstrated that, in addition to the full BM equation, only the revised QUESP equation, can lead to correct results, however only when the $M_i$ is provided (Figure 4). We point out that similar findings on the importance of initial condition were also previously reported by Yuwen et al (41).

It is often stated in the community that both QUESP and QUEST are fully quantitative methods. However, as seen in equations [18 and [24, QUEST can actually determine only $R_{ex}$, whereas QUESP can alter $R_{ex}$ and thus separate $k_b$ and $f_b$. Consequently, QUEST experiments either require the concentration of exchanging species as an input, or the need to scan the whole frequency range and use implicitly the peak width as a second independent access to $k_b$. Actually, single resonant QUEST experiment can never separate $k_b$ and $f_{b,}$ if not also acquired at different saturation powers. Since the separation of $k_b$ and $f_b$ is only possible by QUESP, we recommend that quantitative CEST experiments are performed using variable saturation power. Important to note is that QUESP requires the longitudinal relaxation rate of water $R_{1a}$ for absolute concentration determination. Additionally, we want to point out that for single exchanging pools also on-resonant spin-lock allows for accurate quantification of exchange rates and can provide an alternative measurement to CEST (25).

**ParaCEST results**

The estimated exchange rates for EuDOTAM-Gly (Figure 6) are in line with the previously published results. We obtained $k_b$ between 6000-7600 Hz at 25 °C with the different methods, which matches the originally reported value of 6250 Hz at 25 °C (27). Interestingly, the concentrations obtained from the fits were slightly higher ($f_b$=1.2-1.4 ‰) than actually used in experiments ($f_b$=0.9 ‰), however results were consistent for the BM fit as well as the QUESP methods. For higher temperature, the breakdown of the QUESP methods could be assessed using the underlying equation [21 (Appendix A) of α which is only valid in the narrow-peak-limit $\delta\omega >> \Gamma$; where $\delta\omega$ is the chemical shift difference and $\Gamma$ is the $B_1$ dependent width of the CEST peak (equation [20) that depends on the exchange rate (31). For the paraCEST agent used in this study, we conclude that the exchange rates should be smaller than 10000 Hz for the QUESP/QUEST and Ω-plot methods to be valid at a field strength of 7 T (i.e. the agent is resonating at 55 ppm at 7T, which gives $\delta\omega$ = $7\cdot\gamma\cdot 50$ ppm ≈ $10^5$ $s^{-1}$ and $\Gamma(k=10^4\ Hz, B_1 = 25\ \mu T) \approx 20000\, s^{-1}$).

In principle, the QUESP equation can be extended by the full term for labeling efficiency resulting in the equation [14], which reads according to (31).

$$\alpha = \frac{\frac{\delta\omega_b^2}{\omega_1^2+\delta\omega_b^2}k_b + R_{2b}}{k_b + R_{2b}} \frac{\omega_1^2}{\omega_1^2 + k_b(k_b + R_{2b})} \tag{14}$$

This should potentially extend the convergence interval of the QUESP method for higher exchange rates or smaller chemical shifts, such as salicylic compounds (42,43) or endogenous metabolites (31,44). We want to point out that we neglected $R_{1b}$ and $R_{2b}$ in all evaluations with respect to the fast exchange rates, however, especially for lower temperatures, CEST pool relaxation rates might not be negligible for fitting paraCEST spectra, thus, while $R_{2b}$ is already included (26), the analytical description of the labeling efficiency still needs to be extended by $R_{1b}$ as discussed in the supporting information.

**Optimal $B_1$ and $t_p$ ranges**

When the experiments are set up, the common question arising is which $B_1$ values and saturation times should be used for optimal sampling of QUESP and QUEST experiments. QUESP evaluation becomes easier in steady-state and long $t_p$, but signals can be higher for lower saturation times, especially in the presence of spillover (45). To improve separation of $k_b$ and $f_b$, the $B_1$ values in QUESP need to sample the varying labeling efficiency α (equation [21, Appendix A) as well as possible. This is the case if $B_1$ are sampled around $B_1$=k/γ, e.g. 0.25·k/γ, 0.5·k/γ, 1·k/γ, 1.5·k/γ and 2·k/γ would lead to α=6%, 20%, 50%, 69% and 80%. Of course this requires an estimate of the exchange rate, but it is plausible that sampling around 50% ($B_1$=1·k/γ) of the maximal effect is close to optimal. In principle, two $B_1$ values are enough to solve the equations, but more sampling improves the estimation. As it can be seen in Figure 2b, using too low $B_1$ powers hinders determination of fast exchange rates, and using too high $B_1$ powers hinders determination of slower exchange rates. The same trend is shown in Figure 2f – here MTR shows a plateau, depending on $B_1$, meaning particular MTR($B_1$,$k_b$) regions are almost independent of $k_b$.

The same curve in Figure 2f gives insight into the QUEST measurement setup – $B_1$ values should also be chosen that the expected exchange rate does not fall into the plateau. It is important to note that two solutions for $k_b$ can exist for the same QUEST course, one being left from the optimum and one right from the optimum (Figure 2f). This makes the correct choice of $B_1$ for QUEST even more important. Finally, the saturation time $t_p$ should sample the saturation built-up which is typically below 1-2 x $T_1$. Beyond these rules of thumb, the optimal sensitivity of the CEST sequence can be derived by combining the analytical formula with the approach of Jiang et al. for steady-state sequences (46).

### Radiation damping

We want to point out that data acquisition (especially FID at high magnetic fields) for non-equilibrium initial condition or non-steady state (so especially QUEST) are prone to errors originating from altered $T_1$ relaxation during recover and saturation time due to radiation damping effects (47). As radiation damping and thus the effective $T_1$ depends on the size of the magnetization vector, the QUEST method can be drastically affected by this effect and estimated exchange rates are questionable (see also Supporting Figure S4). We were able to reduce this effect by an order of magnitude using a smaller NMR tube for the shown accelerated sequence (Figure 8).

### 2π or not 2π

When a QUESP dataset or an omega-plot is evaluated, the exchange rates are obtained in units of $\omega_1$, which is rad·s$^{-1}$. Therefore, the question arises whether the final results of $k_b$ have to be converted from rad·s$^{-1}$ to s$^{-1}$, by dividing with 2π. By looking into the fundamental Bloch-McConnell equations [20], one notes that $\omega_1$ and $T_1$, $T_{2,}$ or the exchange rate $k_b$ appear within the same equation, however with different units of rad·s$^{-1}$ and s$^{-1}$, respectively. Thus the value of oscillation constants corresponds directly to the value of the exchange rate in s$^{-1}$, and we conclude that no further division by 2π should be performed to obtain the final value of the exchange rate.

### Spillover effect, multiple pools, pulsed saturation

The quantification methods in this work were only applied in a paraCEST system where direct water saturation is not an issue. Albeit not shown herein directly, all equations based on the inverse metric (i.e. $MTR_{Rex}$ in equations [3 and [8) will still be valid, as long as saturation steady-state is reached (33,39,48). On the other hand, all dynamic solutions will not be directly valid in the case of strongly overlapping direct water saturation. In such case, our methodology can be extended using the QUESTRA approach of Sun et al.(49).

Moreover, pulsed saturation can be incorporated for steady-state conditions as shown previously (32,33). However, when a single offset approach is being used, the interplay of several CEST resonances further confounds the obtained results. Finally, $R_{2b}$ values should generally be taken into account for all the presented approaches, which is easily possible, however in the case of paraCEST agents, these contributions are negligible.

## Conclusions

How to accurately measure exchange rates using CEST? In this article, we have attempted to address the CEST quantification issue by providing revised equations to be used in respective calculations, discussing different theoretical and experimental cases, and finally providing some practical hints. In addition to completing the theory by a missing labeling factor, we were able to accurately estimate exchange rates and extend the analytic models for the case of arbitrary initial magnetization and subsequently non-steady-state saturation. By completing the quantitative description, we could show a methodology that allows for more freedom in choosing the appropriate quantitative CEST experiments, and also to speed up the quantitative experiments without biasing the final results.

# Acknowledgments

The authors thank Dr. Nevenka Cakić for preparing the EuDOTAM-Gly complex. The financial support of the Max Planck Society, German Research Foundation (DFG, grant ZA 814/2-1, support to M.Z.), European Union's Horizon 2020 research and innovation programme (Grant Agreement No. 667510, support to M.Z.) is gratefully acknowledged. X.G. is funded partly from the Department of Health's NIHR Biomedical Research Centres funding scheme to UCL/UCLH.

# Appendix A

CEST results are presented in so called Z-spectra. To obtain a Z-spectrum, the amplitude of the saturated water peak ($M_{sat}(\Delta\omega)$), normalized as a proportion of the water signal in thermal equlibrium ($M_0$), is plotted as a function of the saturation pulse frequency offset from water $\Delta\omega$. The normalized Z-spectrum is described by the function $Z(\Delta\omega)$ given by equation [15].

$$Z(\Delta\omega) = M_{sat}(\Delta\omega)/M_0 \qquad [15]$$

In the case of continuous saturation with a block pulse of amplitude $B_1=\omega_1/\gamma$ and duration $t_p$, the normalized water Z-magnetization $Z(\Delta\omega)$ has been derived previously,(31) and it is given with equation [16], where $Z_i$ is the initial Z–magnetization before saturation and can be written as $Z_i = M_i/M_0$.

$$Z(\Delta\omega, t_p) = Z^{ss}(\Delta\omega) + (\cos^2\theta \cdot Z_i - Z^{ss}(\Delta\omega))e^{-R_{1\rho}(\Delta\omega)t_p} \qquad [16]$$

The measured $Z(\Delta\omega, t_p)$ decays or grows during the saturation module towards a steady–state, given by equation [17].

$$Z^{ss}(\Delta\omega) = \cos^2\theta \frac{R_{1a}}{R_{1\rho}(\Delta\omega)} \qquad [17]$$

With the longitudinal relaxation rate of water $R_{1a}$, the longitudinal relaxation rate in the rotating frame of water $R_{1\rho}$ and the tilt angle of the effective field $\theta = \tan^{-1}(\omega_1/\Delta\omega)$. In the large-shift limit (chemical shift of the CEST pool $\delta\omega_b >> \omega_1, k_b$) and without semi-solid MT, some terms simplify: $\cos^2\theta = 1$, $R_{1\rho} = R_{1a} + R_{ex}(\Delta\omega_b)$ and close to the CEST resonance, the Z-value can be defined with equation [18].

$$Z(\Delta\omega_b, t_p) = \frac{R_{1a}}{R_{1a} + R_{ex}(\Delta\omega_b)} + (Z_i - \frac{R_{1a}}{R_{1a} + R_{ex}(\Delta\omega_b)})e^{-(R_{1a} + R_{ex}(\Delta\omega_b))t_p} \qquad [18]$$

With the exchange-dependent relaxation $R_{ex}(\Delta\omega_b)$ which simplifies into equation [19] for narrow peaks ($\delta\omega_b > \Gamma \approx 2\sqrt{\omega_1^2 + k_b^2}$) to a Lorentzian line centered at the frequency offset relative to the CEST pool $\Delta\omega_b$, with the linewidth $\Gamma$ defined with equation [20], and the value of the label scan, $R_{ex}^{lab} = R_{ex}(\Delta\omega = \delta\omega_b) = R_{ex}(\Delta\omega_b = 0)$, given by equation [21].

$$R_{ex}(\Delta\omega_b) = \frac{R_{ex,LS}^{lab} \ \Gamma^2/4}{\Gamma^2/4 + \Delta\omega_b^2} \tag{19}$$

$$\Gamma = 2\sqrt{\frac{R_{2b}+k_b}{k_b}\omega_1^2 + (R_{2b}+k_b)^2} \tag{20}$$

$$R_{ex}^{lab} = f_b k_b \cdot \alpha = f_b k_b \frac{\omega_1^2}{\omega_1^2 + k_b(k_b + R_{2b})} \tag{21}$$

The reference value at the opposite frequency can often be assumed as 0 according to equation [22], if $\Delta\omega >> \Gamma$.

$$R_{ex}^{ref} = R_{ex}(\Delta\omega = -\delta\omega_b) = R_{ex}(\Delta\omega_b = -2\delta\omega_b) \approx 0 \tag{22}$$

The exchange-dependent relaxation $R_{ex}$ is the most important parameter for CEST effects and it can be used to determine the exchange rate $k_b$ of the exchangeable protons with concentration fraction $f_b$ and transverse relaxation $R_{2b}$. Please note, that compared to $R_{ex}$ defined by Trott and Palmer (50) or Jin at al. (25) the $R_{ex}^{lab}$ term here includes a $\sin^2\theta$ term, so $R_{ex}^{lab} = \sin^2\theta \cdot R_{ex}$. This is necessary to derive the known labeling efficiency α.

The magnetization transfer ratio asymmetry ($MTR_{asym}$) is defined as the difference in signal on either side of the water peak centered at 0 ppm for constant amplitude $B_1$, according to equation [23], where the label and reference normalized z-magnetizations are $Z_{lab} = Z(\Delta\omega = +\delta\omega_b)$ and $Z_{ref} = Z(\Delta\omega = -\delta\omega_b)$ respectively.

$$MTR_{asym} = Z_{ref}(t_p, B_1) - Z_{lab}(t_p, B_1) \tag{23}$$

Using equation [18¸ the $MTR_{asym}$ yields equation [24].

$$MTR_{asym} = \frac{R_{ex}^{lab}}{R_{1a} + R_{ex}^{lab}} + (Z_i - 1)e^{-R_{1a}t_p} - \left(Z_i - \frac{R_{1a}}{R_{1a} + R_{ex}^{lab}}\right)e^{-(R_{1a} + R_{ex}^{lab})t_p} \tag{24}$$

This asymmetry analysis provides a clear representation of the CEST effect assuming that direct water saturation is not overlapping strongly the CEST peak.

Equations [18 and [21 show that two experiments can be designed to quantify exchange rates independently from the water signal, namely quantification of exchange by varying saturation time $t_p$ (QUEST) and saturation power $B_1$ (QUESP).

Equation [24 in steady-state ($t_p >> R_1$) is further simplified to equation [25]:

$$MTR_{asym} = Z_{ref}(B_1) - Z_{lab}(B_1) = \frac{f_b k_b \cdot \alpha}{R_{1a} + f_b k_b \cdot \alpha} \qquad [25]$$

In the case of overlapping direct water saturation, $MTR_{Rex}$ is a useful metric that has been derived previously for steady-state conditions (26) and can be written as equation [26]:

$$MTR_{Rex} = \frac{1}{Z_{lab}(B_1)} - \frac{1}{Z_{ref}(B_1)} = \frac{f_b k_b \cdot \alpha}{R_{1a}} \qquad [26]$$

In vivo applications of CEST are shown to be benefited from the fact that $MTR_{Rex}$ removes $T_2$ and MT dependencies, and hence directly yields measurements of $R_{ex}$ (39). In addition, the plot of 1/ $R_{ex}$ as a function of $(1/\omega_1)^2$ yields a linear function according to equation [21 which can be used as another approach for exchange rate quantification and it is known as the Ω-plot method (see also equations [10 and [11.

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# Figures

**Figure 1:** (a) The sequence diagram of a typical CEST experiment consists of 3 modules: recovery module of duration $t_{rec}$, saturation module of duration $t_p$, and acquisition module of duration TA. (b) Magnetization course during continuous wave (cw) irradiation of amplitude $B_1$: If $t_{rec}<<5\times T_{1a}$, the z-magnetization recovers to $M_i\neq M_0$, if $t_p<<5\times T_{1a}$ the $M_{sat}$ can depend on $M_i$. (c) Magnetization course for far off-resonant irradiation: the course during $t_p$ is also approximately governed by $T_1$ and thus approaches $M_0$. If $t_{rec}+t_p<<5\times T_1$ this value can still be $M_{offres}<M_0$. Given that $Z(\Delta\omega) = M_{sat}(\Delta\omega)/M_0$, the Z notation of the z-magnetization will be used in the rest of the manuscript.

**Figure 2:** Simulated Z-spectra obtained by varying (a) saturation power $B_1$, (c) saturation time, and (e) varying exchange rate. (b,d,f) Comparison of BM simulation (circles) with different analytical QUESP/QUEST formulae (equation [6 dashed line, equation [4 solid line) for varying (b) saturation power, (d) saturation time, (f) varying exchange rate k for $t_p$=3 s. As the labeling efficiency term $\alpha$ was added in the revised formula (equation [4), the two equations match when $k_b<< \gamma B_1$ and thus when full labeling is achieved. In the case of weak labeling ($\alpha<0.5$) the revised equation yields much better match with the BM simulation. The curves in (b) for 1000 Hz match both equations [4 and [6 also for $\alpha<<1$; this can be explained by the fact that $k_b\cdot f_b=0.135<R_{1a}=0.33$, hence the minor influence of the $\alpha$ terms in this case.

**Figure 3:** Different QUESP methods applied to simulated data for $f_b = 1.35\times10^{-4}$, $k_b$=1000 Hz (left) and $k_b$=9000 Hz (right). (a,b) The original QUESP equation in steady state $MTR=k_b\times f_b\times\alpha /(R_{1a}+k_b\times f_b)$ (blue solid line, equation [6) and the revised QUESP equation in steady state $MTR=k_b\times f_b\times\alpha /(R_{1a}+k_b\times f_b\times\alpha)$ (red dashed line, equation [4). Although both equations fit the data, the revised equation yields much better estimates for exchange rates, especially for lower labeling (faster exchange). (c,d) QUESP method in steady state using the inverse metric approach (green dashed line, equation [9) $MTR_{Rex}=k_b\times f_b\times\alpha/R_{1a}$. (e,f) Ω-plot method in steady-state (equation [10).

**Figure 4:** QUEST fitting with original and revised equation (equations [6 and [4, respectively) for (a) $k_b$=1000 Hz, and (b) $k_b$=9000 Hz. Estimation is improved by the revised formula especially for higher exchange rates. Note: when a starting value of $k_b$=4000 Hz was chosen, the fit for MTR ($k_b$=1000 Hz) yielded a completely wrong estimation, because of the two possible values of $k_b$ with the same MTR (see Figure 2f).

**Figure 5:** (a,b) Different QUESP evaluations applied to non-equilibrium simulated data for defined $Z_i$=0.3, short $t_p$=3 s, relative concentration $f_b = 1.35\times10^{-4}$, and exchange rates (a) $k_b$=1000 Hz, and (b) $k_b$=9000 Hz. Generally, all the formulae fit the QUESP data, however, both equations [6 and [4 lead to wrong parameter estimations. The fit of the data leads to meaningful estimations only if the initial magnetization is properly incorporated in the model (equation [12). (c,d) Different QUEST evaluations applied to non-equilibrium simulated data for defined $Z_i$=0.3, relative concentration $f_b = 1.35\times10^{-4}$, and exchange rates (c) $k_b$=1000 Hz, and (d) $k_b$=9000 Hz. Equation [12] (red line) is able to fit the data with good exchange rate estimation; again, the models that do not incorporate the initial magnetization fail in predicting the correct exchange rates.

**Figure 6:** Different steady-state QUESP methods applied to experimental data T=13.4 °C (left) and T=23.8 °C (right). (a,b) Multi-$B_1$-Z-spectral data (dots) fitted with the BM equation (solid lines), showing good match with small residuals (dotted lines at $Z\approx0$). (c,d) Fitting results according to the original QUESP equation [6, showing underestimation of the exchange rates, whereas better estimations are achieved using the revised QUESP equation [4, or (e,f) the Ω-plot method.

**Figure 7:** Comparison of steady state QUESP and Ω-plot methods with full BM fit results (data points) together with corresponding temperature fits obtained using Arrhenius equation (equation [13, lines). (a) Temperature range between 12 °C and 30 °C: here both Ω-plot and revised QUESP methods yield reasonable results close to the result obtained from BM fit. (b) Temperature range from 12 °C to 50 °C: here only the BM fit shows good accuracy, whereas the QUESP method deviates already above $10^4$ Hz and the Ω-plot method deviates above $2\cdot10^4$ Hz. The $k_c$ values obtained according to the BM equation match well the Arrhenius fits for both temperature ranges.

Figure 8: Different QUESP methods applied to experimental data T=13.4 °C (left) and T=23.8 °C (right) for initial conditions $Z_i$<1.=0.87 (a,), and $Z_i$=0.77 (b)). Multi-$B_1$-Z-spectral data (dots) fitted with the BM equation (solid lines), showing good match with small residuals (dotted lines at $Z \approx 0$). (c,d) Fitting results according to the original QUESP equation, showing underestimation of the exchange rates, whereas better estimations are achieved using the revised QUESP including $Z_i$ (equation [12), or (e,f) the Ω-plot method.

**Supporting Figures**

Figure S1: $MTR_{asym}$ of simulated data (circles) together with analytic solutions of equation [2] (solid lines) in the case of normalization by $M_0$ (a,b,c) and normalization by $M_{offres}=0.71\times M_0$ (d,e,f). In figures (g,h) and (i) both the data and the analytical formula (dashed line, equation [3]) is normalized by $M_{offres.}$

Figure S2: $MTR_{asym}$ of simulated data normalized by individual $M_{offres}(t_{p)}$ (circles) together with differently normalized analytic solutions (lines). (abc) Solid lines show eq.(2) with M0 normalization. (def) Dashed lines show equation [3] with $M_{offres}(3s)=0.71\cdot M_0$ normalization. (ghi) Dash-dotted lines show equation [4] with individual $M_{offres}(t_p)$ normalization.

Figure S3: $MTR_{asym}$ of simulated data normalized by individual $M_{offres}(t_{p)}$ (circles) with the original eq. (1) without $Z_i$ (a,b,c). In Figure (d,e,f) equation [1] was normalized manually by a factor 1.19 to show that the analytic solution (equation [1]) yields a relatively good match to the data.

Figure S4: $T_1$ inversion recovery data without (red lines) and with gradient (blue lines) applied during the inversion time. By applying the gradient radiation damping is avoided. Comparison of (a) and (b) reveals that going from a 5mm tube to a 1.6mm tube solves this issue.