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Guidelines for the Use of Deuterium Oxide (D_2O) in ^1H NMR Metabolomics

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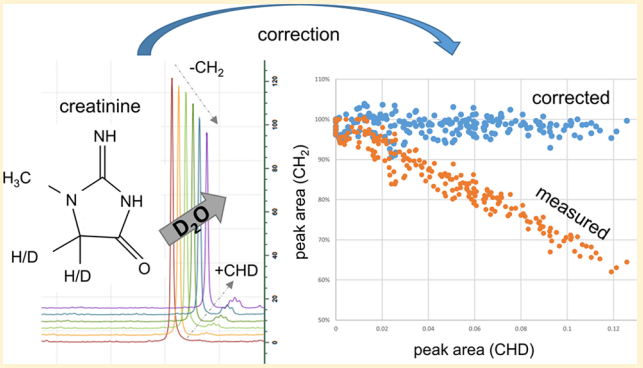
Supporting Information

ABSTRACT: In metabolomics, nuclear magnetic resonance (NMR) spectroscopy allows to identify and quantify compounds in biological samples. The sample preparation generally requires only few steps; however, an indispensable factor is the addition of a locking substance into the biofluid sample, such as deuterium oxide (D_2O). While creatinine loss in pure D_2O is well-described, the effects of different D_2O concentrations on the signal profile of biological samples are unknown. In this work, we investigated the effect of D_2O levels in the NMR buffer system in urine samples, in dependence on dwell time and temperature exposition. We reveal a decrease of the urinary creatinine peak area up to 35% after 24 h of dwell time at room temperature (RT) using 25% (v/v) D_2O, but only 4% loss using 2.5% D_2O. ^1H, inverse-gated (IG) ^{13}C, DEPT-HSQC NMR, and mass spectrometry (MS) experiments confirmed a proton−deuterium (H/D) exchange at the CH_2. This leads to underestimation of creatinine levels and has an extensive effect when creatinine is used for normalization. This work offers a sample stability examination, depending on the D_2O concentration, dwell time, and temperature and enables a method to correct for the successive loss. We propose an equation to correct the creatinine loss for samples prepared with various D_2O concentrations and storage temperatures for dwell times up to 24 h. The correction function was validated against an external data set with $n = 26$ samples. To ensure sufficient creatinine stability in future studies, we suggest that a maximum of 10% D_2O should be used at 4 °C or 2.5% D_2O at RT, respectively.



Metabolomics aims to comprehensively characterize (identify and quantify) metabolites in biological fluids and tissues and to study underlying pathways and biological implications.[1−3] Metabolome research offers the possibility to reveal valuable knowledge, which helps to address various aspects, including personalized medicine, the estimation of environmental or dietary impacts on individuals, and biomarker discovery.[3−5]

Urine is a widely used biofluid, because of its availability in large quantities and the noninvasiveness of sampling.[6,7] Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the key techniques used in urine metabolomics.[5] The former technique benefits from high robustness and quantitation in nontargeted analysis.[8]

Standard operation procedures (SOPs) for NMR-based metabolomics reached some level of agreement, but still some variations exist, in terms of phosphate buffer concentration, concentration of D_2O, and addition of chemicals for positional noise reduction.[7−10] While phosphate buffer is added to maintain a constant pH of 7.4, D_2O is necessary to ensure a sufficient locking for stabilization of the magnetic field strength and to avoid ^1H containing solvents that would unnecessary inflate the NMR spectrum.[2,7,11] Keeping measurement conditions constant is essential in metabolomics because of a general large sample quantity and high-throughput measurements over several hours using autosampling devices.

In addition to the variation in sample preparation procedures, urine as a biosample matrix poses the challenge of handling inherent urinary dilution. Several methods are available, with the most common being probabilistic quotient normalization (PQN)[12] and normalization to urinary creatinine. Creatinine is a breakdown product of creatine phosphate in muscle tissue. It is removed from the body by the kidneys through urinary excretion and known to be a useful marker for renal function.[13,14] If no renal dysfunction exists, creatinine is excreted at a constant rate via urine in 24 h and therefore can be used as normalization factor to correct urinary dilution.[15,16] Furthermore, creatinine is an important bio-

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marker especially in investigations regarding kidney diseases and renal function.[17]

Yet, D_2O is known to affect hydrogen−deuterium exchange in creatinine, especially in freeze-dried samples, which are reconstituted in pure D_2O.[18] In this case, the CH_2 creatinine peak disappears or is reduced, which leads to inaccurate quantification.

In this study, we investigated the effects of D_2O concentrations in urine buffers on metabolites with a focus on creatinine. Creatinine underwent a conversion over time, which resulted in a decrease of the creatinine peak at δ 4.06 ppm and an increase of a triplet upfield (δ 4.04 ppm). We describe the underlying mechanism and propose an optimal sample handling guideline for urinary NMR metabolomics to ensure stable creatinine quantification for high-throughput measurements.

MATERIALS AND METHODS

Sample Preparation. Urine samples from two distinct groups were used: group A consisted of pooled spot urine from 5 healthy individuals, whereas group B contained 26 samples from a previous intervention study, as described in the 2015 work of Lagkouvardos et al.[19] All experiments concerning the impact, mechanism, and mathematical correction of the deuterium oxide effect on urine were performed on samples from group A. These were collected in 50 mL polypropylene tubes (Falcon), pooled, and aliquoted into volumes of 150 μL for analysis. A second dataset from group B was used for validation of the correction equation. Each volunteer provided written informed consent.

Samples were stored at −80 °C until analysis. Aliquots were thawed on ice, homogenized by vortexing, and transferred into vials containing buffer solution. A 1.5 M K_2PO_4 solution (pH 7.4) was used as a buffer that contained 0.1% trimethylsilylpropionic acid (TSP) in either 10% D_2O (buffer I) or 100% D_2O (buffer II). Buffers I and II were mixed to obtain required total D_2O concentration for analysis of final D_2O concentrations from 2.5% to 25%. Buffer solutions and urine were mixed in a ratio of 1:3 (50 μL buffer and 150 μL urine) and centrifuged at 4 °C for 10 min at 13 000 *g*. A quantity of 180 μL of supernatant was transferred into 3-mm NMR glass vials. For elucidation of the mechanism, 100 μL of a 0.33 M creatinine standard solution in H_2O (∼7.5 mg/sample) was diluted in 50 μL of buffer I and 50 μL of H_2O, resulting in a total D_2O concentration of 2.5%. Equivalently, a sample with a final concentration of 50% D_2O was prepared by mixing 100 μL of the standard solution in 50 μL of buffer II and 50 μL of D_2O. The standard samples were left at RT at least for 24 h to ensure that equilibrium is reached.

The impact of creatinine loss was estimated using 2.5%, 10%, and 25% D_2O samples. Between sample preparation and measurement, samples were stored at RT and 4 °C. RT samples were prepared once and remeasured after the defined time increments, whereas cooled samples were prepared 13 times and, for every increment, a new sample was measured to exclude the effect of temperature increase during acquisition.

For calculation of the correction equation, samples were prepared from pooled urine. D_2O concentrations in these samples were adjusted to 2.5%, 5%, 10%, 15%, 20%, and 25%, respectively. Samples were measured in increments of 2 h from t = 0 h to t = 24 h. Between sample preparation and measurement, samples were stored at RT. All sample preparation steps were performed on ice until analysis.

NMR Instrumentation and Data Processing. Urine samples were analyzed on a Bruker 800 MHz spectrometer that was operating at 800.35 MHz and was equipped with a quadrupole inverse cryogenic probe (Bruker BioSpin); the 90° pulse was set to 14 μs. Sixteen scans were recorded into 64 K data points with a spectral width of 16 ppm. As a quality marker, the peak width at half-maximum for the TSP peak was monitored and spectra with a peak width at half-maximum of >1.0 Hz were excluded. All spectra were acquired at 300 K. One-dimensional proton spectra were acquired using a standard 1D-pulse sequence with water suppression (noesygppr1d) during an RD of 4 s, an acquisition time of 3 s, and a mixing time (tm) of 200 ms. To avoid integration of neighboring signals, integration boundaries of ±8.5 Hz around the centroid value were set.

One-dimensional (1D) carbon spectra were acquired using an inverse-gated (IG) decoupling pulse (zgig) with proton decoupling (WALTZ-16) during the recycle delay (RD) of 58 s to eliminate a nuclear Overhauser effect (NOE), a 90° pulse for ^{13}C at 13 μs, a decoupler pulse at 12 μs, and a decoupler power level at 1.11 dB. Multiplicity edited HSQC spectra were recorded using a DEPT-HSQC (distortionless enhanced polarization transfer heteronuclear single quantum coherence) pulse sequence (hsqcedetgpsisp2.2). Spectral width was set to 13 and 50 ppm in the proton (F2) and carbon (F1) dimensions, respectively. For each 2D spectrum, 5578 × 3072 data points were collected using 2 scans per increment with an acquisition time of 0.25 s and 16 dummy scans.

Acquisition and processing were performed using TopSpin 3.5 software (Bruker BioSpin). Free induction decay (FID) were multiplied by an exponential function corresponding to line broadening of 0.3 Hz prior to Fourier transformation. All spectra were manually phased, baseline corrected and calibrated to TSP (δ TSP = 0 ppm) before exporting into Matlab software (R2011b; Mathworks) for further data processing.

The water region was removed (δ 4.6−5.0 ppm). Spectra were aligned using a recursive segment-wise peak alignment (RSPA) algorithm.[21] Orthogonal partial least-squares (OPLS) analysis was performed as described by Cloarec et al.[22] Integrals were calculated using trapezoidal numerical integration. Local baseline correction was performed by generating linearly spaced vectors between integration boundaries and subtracting the resulting integrals from peak integrals. Negative peak integrals of the deuterated creatinine triplet (i.e., in the absence of deuterated creatinine) were set to zero. All integral areas were normalized to the corresponding TSP peak area as an internal standard. For investigation of creatinine loss over time, measured CH_2 integrals were expressed in % of CH_2 peak area recorded at t = 0 (CH_2/CH_{2i} [%]).

MS Measurements. Analysis of the isotope distribution was performed using a maXis qTOF-MS equipped with an APOLLO II electrospray ion (ESI) source (Bruker Daltonics). Samples were measured via direct injection and in electrospray positive mode. Source settings were the same as elsewhere described:[23] nebulizer pressure = 2 bar, dry gas flow = 10 L/min, dry gas temperature = 200 °C, capillary voltage = 4.5 kV, end plate offset = +500 V, mass range = m/z 50−1500.

RESULTS AND DISCUSSION

To initially investigate the impact of sample preparation conditions on urine samples, we measured pooled urine samples with altering D_2O after an equilibration time of 24 h

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after buffer contact. In Figure 1, an overlap of six urine spectra with altering D_2O concentrations shows a clear decrease in

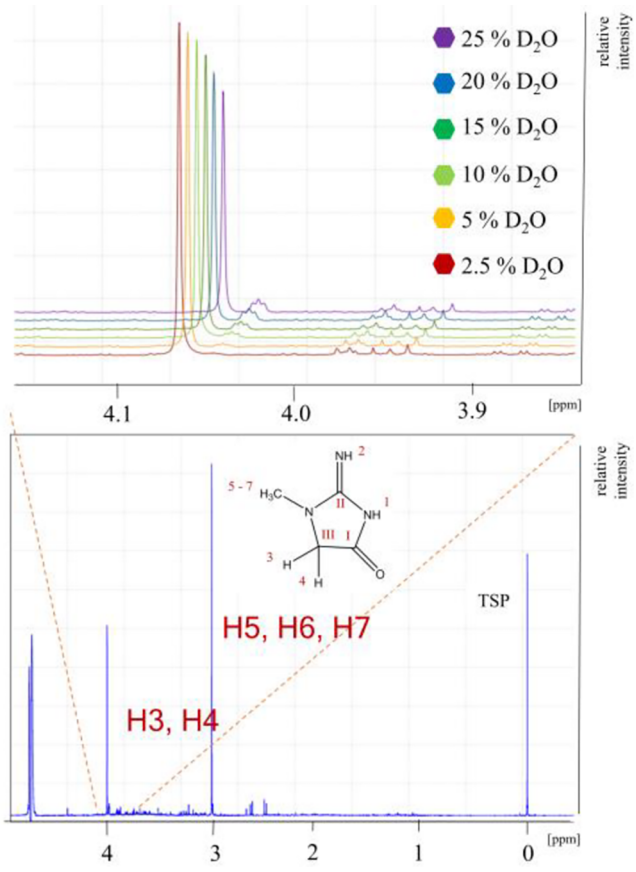

Figure 1. Proton spectra (−0.1−5.5 ppm) of creatinine standard in H_2O/D_2O and buffer with peak annotation to creatinine structure and enlargement of CH creatinine peak area as stacked plot with D_2O concentrations ranging from 2.5% (red) to 25% (purple).

creatinine CH_2 peak intensity after 24 h and an increase in an upfield triplet, depending on D_2O concentration. At a D_2O concentration of 2.5%, no triplet was observed, i.e., the D_2O concentration was too low to induce an effect. To systematically investigate further effects of D_2O over time at RT, besides creatinine, we analyzed samples under the two extreme D_2O concentrations (2.5% and 25%) every 2 h for 24 h, taking 2.5% D_2O as a control. No other signals were found (threshold correlation coefficients of $R^2 > 0.5$). Yet, several urine metabolites are known to be susceptible to proton−deuterium exchange, such as histidine,[24] which was not seen here. Our results suggest that, under the sample preparation conditions of 25% D_2O within 24 h, no other metabolites are affected by the H-D exchange. Therefore, the following evaluation focuses on the observed effects on creatinine.

The main issue with a decrease in creatinine peak area is derived from the usage of creatinine as a normalization factor and its utilization as an important marker for renal activity. To circumvent this issue, alternatively to the CH_2 peak, the CH_3 moiety could serve for creatinine quantification. The standard deviation of peak area of CH_3 was very low (<2%). However, a 2D-HSQC spectrum of a QC sample from a clinical study (for details, see the 2018 work of Gil el al.[20]) revealed overlap in the CH_3 peak area but not for the CH_2 peak (see Figure S1A in the Supporting Information). This overlap is derived from 1,1-dimethylbiguanide (metformin). Metformin is a first-line medication for type-2 diabetes. Type-2 diabetes had a global total prevalence of 8.4% in 2014, ranging from 7.3% to 13.7%, depending on the region,[25] and is therefore expected to cause substantial problems, especially in epidemiological studies or studies that include diabetes patients. Selected ^1H NMR spectra of type-2 diabetes patients highlight this problem (Figure S1B). For these spectra, the CH_3/CH_2 peak integral has a standard deviation of 30%. Therefore, we concluded that the CH_3 peak is not suitable for creatinine quantification.

Elucidation of H/D-Exchange Mechanism. As suggested by Leibfritz et al.,[18] we hypothesized the cause of this creatinine conversion to arise from a H/D exchange. We examined the underlying mechanism by a combination of (A) solvent-suppressed ^1H NMR for the quantitative estimation of creatinine degradation, (B) inverse-gated (IG) ^{13}C NMR to study changes in the chemical shift due to proton−deuterium exchange and splitting patterns from carbon-deuterium bonds. (C) Multiplicity edited $^1H-^{13}C$-HSQC (DEPT-HSQC) were recorded to link the features revealed by the individual experiments together. This confirmed a D_2O-dependent proton−deuterium exchange at the (3,4)-position (see Figure 2). Neither the addition of potassium fluoride (KF) nor the variance of the phosphate concentration influenced proton−deuterium exchange. However, as expected, the proton−deuterium exchange did not occur in the complete absence of phosphate (data not shown). A decrease of the CH_2 creatinine peak occurs simultaneously with the increase of the monodeuterated (CHD) peak. (IG) ^{13}C spectra allowed us to quantitatively study carbon nucleotides without NOE and uncover a triplet for monodeuterated (III*) ^{13}C and a quintet for polydeuterated (III**) ^{13}C. To investigate the extent of CHD and CD_2 formation under relevant operating conditions, we recorded an IG ^{13}C spectrum of human urine with 25% D_2O. As expected, monodeuteration occurred, but the formation of double deuteration was below a S/N ratio of 3 (see Figure S4 in the Supporting Information).

Pattern splitting occurred because of different nuclear spin systems and proton decoupling ($2NI + 1$, with $I(H) = 1/2$, $I(D) = 1$, and N being the number of nuclei, no splitting for protons), resulting in a singlet for CH_2, a triplet for CHD, and a quintet for CD_2, respectively. Equivalent splitting patterns were found for CH_2 and CHD peaks in DEPT-HSQC-spectra (Figure 2), including a multiplicity inversion for the single resonating proton in the CHD peak.

To confirm the elucidated mechanism, high-resolution electrospray ionization−mass spectrometry (ESI-MS) was used as an orthogonal analytical method to NMR. The proton−deuterium exchange was verified for the 50% D_2O stored for 48 h, after applying positive ESI mode (Figure 3). The spectrum clearly shows the presence of all three states (m/z 114.069 for $[C_4H_7N_3O+H]^+$, 115.076 for $[C_4H_6DN_3O+H]^+$, and 116.081 for $[C_4H_5D_2N_3O+H]^+$. As expected, the H/D-exchange did not occur at the CH_3 of creatinine (δ 3.05) signal in creatinine.

Impact of the H/D Exchange on the Creatinine CH_2 Peak Area under Different Conditions. The described H/D-exchange leads to a loss in CH_2 creatinine peak area. In this work, we investigated to which extent sample preparation (i.e., D_2O concentration of the buffer) and measurement conditions (i.e., temperature during dwell time) affect the resulting peak area. Six different conditions were examined regarding their CH_2 peak area stability over 24 h. We chose

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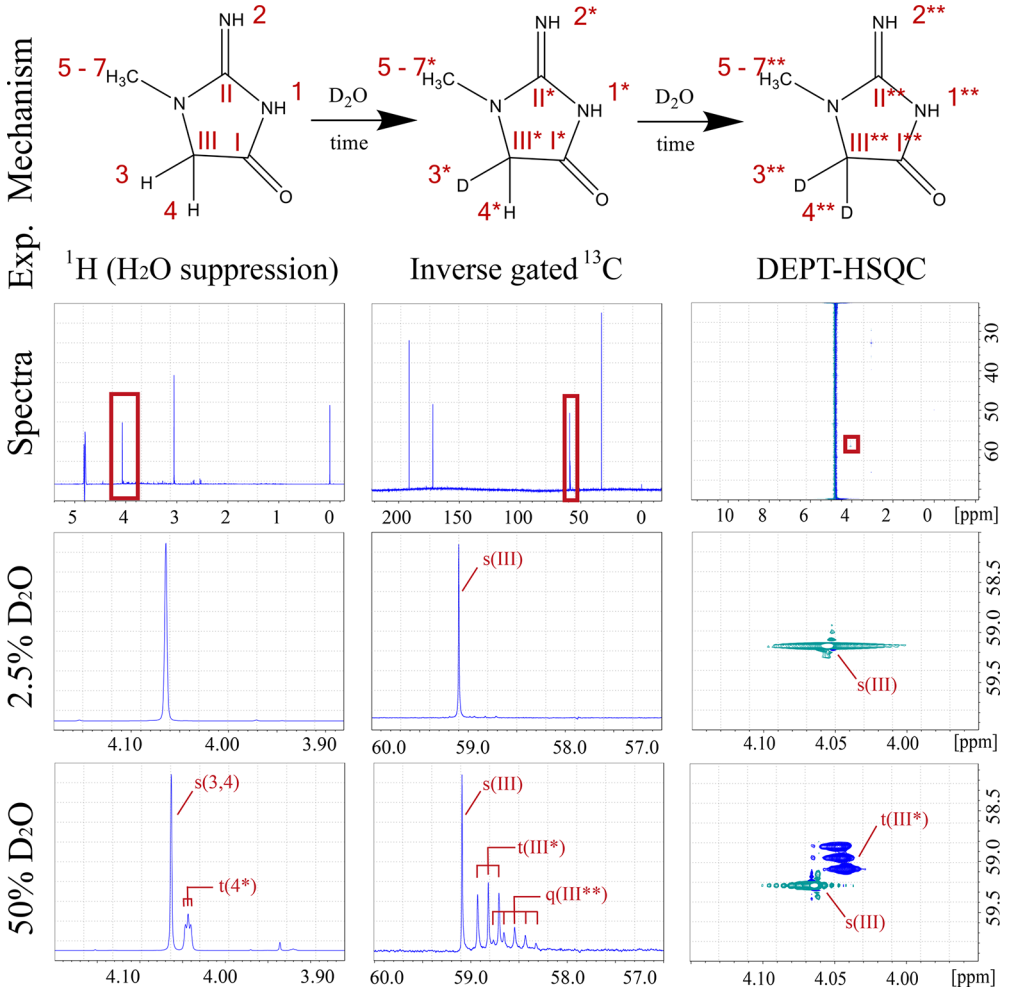

Figure 2. Mechanism of H/D exchange in creatinine with annotation of protons and carbons; ^{1}H, (IG) ^{13}C, and DEPT-HSQC spectra with enlargements of the creatinine peak region and assignments of peaks for 2.5% and 50% D_2O samples 48 h after buffer addition.

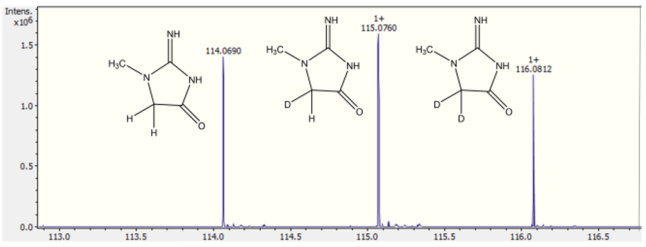

Figure 3. Positive ESI-MS spectrum; enlargement of creatinine including annotation of the different deuteration states (m/z 114.069 for $[C_4H_7N_3O+H]^+$, 115.076 for $[C_4H_6DN_3O+H]^+$, and 116.081 for $[C_4H_5D_2N_3O+H]^+$.

three different D_2O concentrations: 2.5% D_2O as minimal D_2O concentration, 10% D_2O as recommended in widely used urine NMR protocols,[10] and 25% because this sample preparation (≥25%) was used in several previously published studies.[7,26] Samples were kept at RT and 4 °C to cover the conditions of an availability of a cooled autosampler versus analysis at RT.

Prior to the analysis of creatinine conversion, a general estimation of accuracy and robustness was performed, resulting in a relative standard deviation (RSD) of <1% for multiple measurements of the same sample (n = 24) and up to ∼10% variation for measurements of identical samples prepared multiple times and measured on different days (n = 24). This originates from various impact factors on the overall technical error (sample preparation, analytical error, spectral processing, and peak integration variability). Since temperature-controlled time-course measured samples (i.e., 4 °C) were individually prepared and 25 °C samples were prepared only once, the results of the cooled samples are expected to result in a larger inherent variability. Considering this variability, we set an acceptance level for values to be true to ±5% of the CH_{2i} peak area (t = 0).

In Figure 4, we show the impact of D_2O concentrations at RT and 4 °C for dwell time up to 24 h. At RT (Figure 4A), only samples containing 2.5% D_2O are sufficiently stable to allow 24 h of measurements, whereas 10% and 25% D_2O show losses up to 14% and 35% of the initial peak area, with losses of >5% after 4 and 0 h. For cooled samples (Figure 4B), the decrease in peak area is of lesser extent, but still significant: 2.5% and 10% D_2O concentration showed to be sufficiently stable for 24 h, whereas samples containing 25% D_2O showed significant decrease after 8 h. In summary, the availability of a 4 °C cooled autosampling device allows for the use of 10% D_2O, while analysis at RT needs minimization of the D_2O content to no more than 2.5%.

Correction Equation to Compensate Creatinine Loss. In order to use datasets that were analyzed under suboptimal conditions, we went on to investigate the possibility of

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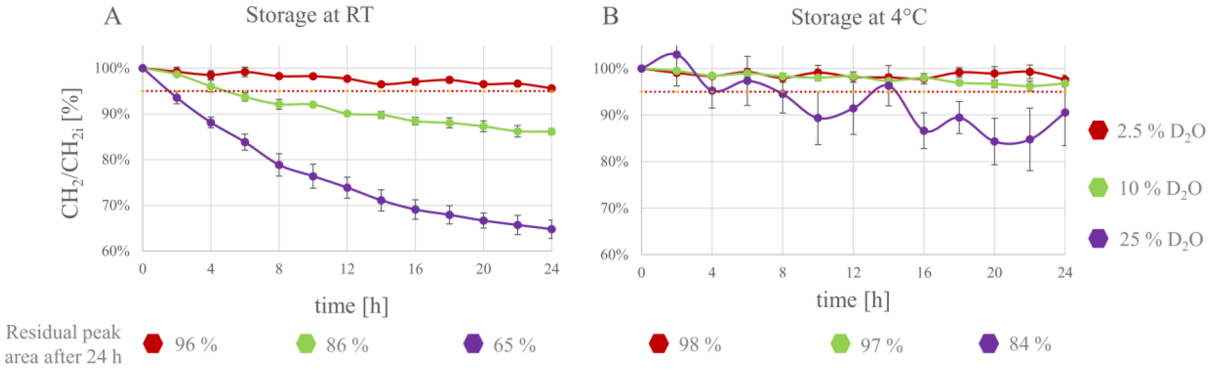

Figure 4. Ratio of measured creatinine CH_2 integral area over time relative to initial creatinine CH_2 for D_2O concentration of 2.5%, 10% and 25% with σ-error bars, dashed lines indicate acceptance limits (100% ± 5%) over 24 h. Data points represent mean values from n = 4 measurements, standard deviations are shown as error bars; residual creatinine peak areas are shown for all conditions. (A) Samples at RT show a strong decrease in the creatinine CH_2 peak area. The strongest effect is visible for 25% D_2O, and only the condition 2.5% D_2O is relatively stable. (B) Storage temperature at 4 °C allows stability of the CH_2 creatinine signal for both 2.5% and 10% D_2O.

correcting creatinine, based on the remaining creatinine CH_2 singlet peak and the emerging CHD triplet.

Using the complete dataset from group A (n = 214), we found a linear correlation (R^2 = 0.94) between the relative change in CH_2 and CHD peak integrals to the initial CH_{2i} integral (Figure 5). The equation, as obtained by linear fitting

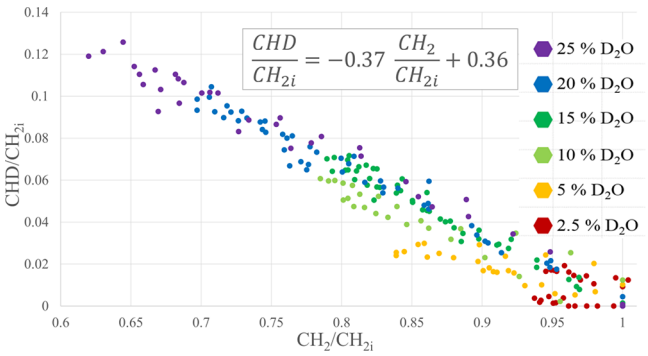

Figure 5. Linear correlation of CHD and CH_2 peak areas after normalization to CH_{2i} peak area at t = 0 h, color-coded by D_2O concentration; the equation shows linear approximation (R^2 = 0.94).

(Figure 5), can be converted and utilized to estimate initial values at t = 0 (CH_{2i}), based on CHD and CH_2 peak areas:

$$CH_{2i} \approx 2.8\mathrm{CHD} + CH_2$$

This equation allows one to estimate the initial creatinine concentration in already analyzed samples, based on the peak integral of the residual creatinine peak (CH_2) and its conversion product, the emerged deuterated creatinine peak (CHD). We hypothesized the empirically found factor of monodeuterated peak area results from two aspects: (1) the relaxation time of hydrogen neighbored to deuterium is larger than hydrogen alone and (2) the CHD peak originated from one instead of two hydrogen atoms since deuterium is ^{1}H NMR invisible.

Indeed, an inversion−recovery T_1 experiment revealed that T_1 relaxation times change from 2.0 s for undeuterated creatinine to 5.8 s for monodeuterated creatinine (see Figures S2 and S3 in the Supporting Information). This results in a significant loss in signal intensity when recycle delays and acquisition times are kept rather short. This signal loss can be corrected by applying the formula for the compensation factor used in the 2017 work of Maitre et al.,[27] which results in a factor of 1.4. Together with the stoichiometric correction of the number of hydrogen atoms, this explains the factor of 2.8 presented herein.

Application of the Correction Equation. We applied this correction to the training dataset used for calculation of the equation (n = 214) and an independent test dataset (n = 26) in order to compare the gained improvement for creatinine quantification (see Figure 6).

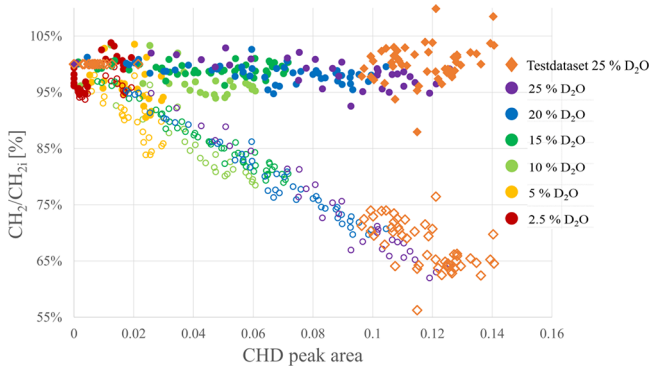

Figure 6. Comparison of dataset A with n = 214 samples for 24 h measurements with (filled circles) and without (empty circles) application of correction including graphical distribution plotted over CHD peak area and for the independent test dataset B with n = 26 samples for t = 0, 12, and 24 h with 25% D_2O with (filled rhombus) and without (empty rhombus) correction.

Table 1 shows creatinine peak areas before and after application of correction for datasets A and B. Remarkably, the result was achieved for different D_2O concentration and independent of time. No systematic error toward D_2O concentration was observed in dataset A. This allows application of the correction for different D_2O concentrations in the buffer and without knowledge of the dwell time (i.e., sample preparation to time of analysis). Potential variation can be introduced by independent overlays of signals in the region of the triplet area. This result also suggests that other degradation and conversion effects are negligible under the investigated conditions of up to 24 h dwell time, RT, and a maximum of 25% D_2O as mean values remain within the accepted error level.

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Table 1. Comparison of 24 h Measurement with and without Application of Correction for Datasets A and B

	Dataset A(n = 214)		Dataset B(n = 26)	
corrected	no	yes	no	yes
$\bar{x}$	85.6%	98.4%	78.6%	100.9%
$\tilde{x}$	85.9%	98.8%	71.0%	100.9%
min	62.0%	90.6%	56.2%	88.4%
max	100.4%	103.8%	100.0%	110.3%
n in ±5% CH_{2i}	21.5%	91.6%	33.8%	93.5%
n in ±10% CH_{2i}	36.9%	100.0%	33.8%	97.4%

This result shows a significant correction of the creatinine peak, exclusively based on the CH_2 and CHD peaks in the acquired spectra.

CONCLUDING REMARKS

In this study, we determined the effect of adding D_2O as buffer solution on metabolite measurements in NMR spectroscopy with a focus on urine as a test matrix. We highlighted that creatinine rapidly undergoes conversions by H/D exchange in contact with D_2O. This leads to underestimated creatinine levels in NMR studies and has an extensive effect when creatinine is used for normalization. Especially in clinical studies, creatinine is a significant marker for renal function; therefore, accurate values are essential for precise data interpretation. As metabolomics studies are generally based on large sample quantities, measurements are executed over several hours, utilizing autosampling devices, and therefore enable the successive creatinine loss. In this study, we introduced a recommendation to address this issue and provide a guideline for future NMR metabolomics studies.

Our results show the importance of sample storage at low temperatures (i.e., 4 °C) prior to analysis, to minimize the creatinine-conversion effect to <5% for at least 24 h. This guideline should be considered for future study designs. In the absence of a cooled device, where measurements are executed at RT, a reduction of D_2O to 2.5% reduces the loss in creatinine peak area to <5% in 24 h.

For already completed measurements under suboptimal conditions, the correction factor introduced here can be applied to correct for loss in integral areas.

The findings in this study show the importance of well-defined and tested standardized operating procedures and sample preparation methodology for urinary NMR metabolomics to produce accurate and significant biological results. Although our application is limited to urine, an adaption to other sample matrices may be of interest for further investigations.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b01580.

Superposition of creatinine CH_3 signal with metformin in 2D-HSQC and ^1H spectra (Figure S1); urine spectra from inversion recovery experiment (Figure S2); determination of T_1 relaxation times for CH_2 and CHD in creatinine (Figure S3); IG ^{13}C for estimation of CD_2 occurrence under real measurement conditions (Figure S4); experimental details and graphs (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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