Instrument validation of the ORYL F1 using mixtures of H_2O/D_2O - Calibration with molecular level sensitivity

Nathan Dupertuis, Orly Tarun, Kaspar Cottier*

**ORYL Photonics SA, Route de la Corniche 5B, 1066 Epalinges, Switzerland*

Abstract

Second Harmonic Scattering (SHS) is a novel technique for studying mechanisms of solubility, aggregation and molecular self-assembly in dipolar solvents. Its application has been limited so far to photonics laboratories with access to tailor made setups on optical benches in a laser class 4 safety laboratory. Here, we report validation experiments obtained using the new ORYL F1, the first commercially available microtiter well-plate-based laboratory SHS instrument. Using H_2O/D_2O mixtures as reference samples, we showcase its high throughput and high accuracy performance at very low sample consumption.

Water is a liquid essential for biological phenomena due to its unique hydrogen-bond (H-bond) network. Substituting hydrogen in light water (H_2O) with deuterium in heavy water (D_2O) profoundly affects its physical properties. These changes arise from nuclear quantum effects that modify intermolecular interactions. SHS is highly sensitive to molecular ordering, showing 40% higher intensity for D_2O in SSS polarization combination, linked to its stiffer H-bond network. D_2O/H_2O mixtures provide a robust calibration for SHS with linearity across angular integration ranges, enabling broad applicability to different setups.

By benchmarking against the state-of-the art, optical-bench angular resolved SHS, with NMR tubes as sample holder in a goniometric configuration, we show that measurement quality of the calibration samples of H_2O /D_2O is retained between the two setups, with the advantage of ORYL's bench-top instrument measuring at a 200x higher throughput and using 10x less sample.

Keywords

- Water
- Heavy water
- Calibration
- Scattering
- Molecular sensitivity
- Molecular ordering
- High-throughput
- Well-plate

Introduction

The liquid state is very important in life sciences as it is at the basis of many biological processes. Among liquids, water is one of the most ubiquitous and crucial, hence earning the qualification of "liquid of life".

The H_2O molecule is made of one oxygen atom in which two hydrogen atoms are covalently bonded with an angle of 104.5° that grants it a dipolar moment as shown in Figure 1. Water molecules are prone to form hydrogen-bonds (H-bonds) with molecules containing electronegative atoms like the oxygen of the other water molecules.

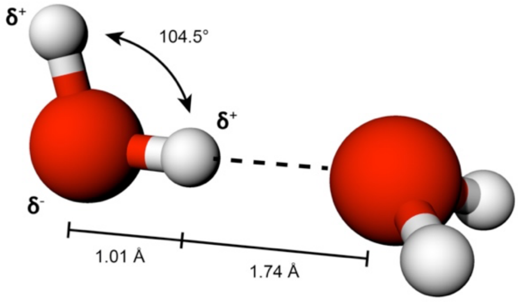


Figure 1. Schematic of two molecules of H_2O with the axis of hydrogen bonding in dashed line, and key geometric features highlighted.

Water is very sensitive to nuclear quantum effects. Here we focus on D_2O, called "heavy water" where deuterium (D) ^2H atoms replace the lighter hydrogen (H) ^1H atoms. Such a simple and limited change in composition has a profound impact on the behavior of liquid D_2O. While some of the properties of H_2O and D_2O are still similar (same dipolar moment, dielectric constant), other properties are dramatically different such as dynamic viscosity, slower dynamics, vibrational changes, etc)[1]. In particular, the hydrogen bond network of D_2O is stronger than in H_2O.

More generally, liquids and solvents are shaped by intermolecular interactions that affect their structure and their behavior with any other molecule, solute or particle. In order to establish that a measurement method has good molecular level sensitivity, and is able to probe subtle changes in intermolecular interactions in liquids at molecular level sensitivity, one needs a proper liquid model. Mixtures of H_2O and D_2O match this requirement as they are profoundly impacted by nuclear quantum effect. They are also stable, safe, and relatively abundant solvents, which makes them excellent candidates to establish a robust calibration method for instrument setups measuring these intermolecular interactions, and to compare their performance.

In this work we outline an experimental approach using Second Harmonic Scattering (SHS) to study mixtures of H_2O and D_2O and to detect changes in liquid samples at the molecular level. We use the ORYL F1 from ORYL Photonics to measure SHS from liquid samples in well-plates, and compare it to a state-of-the art optical bench goniometric SHS setup. We show that the benefits of second harmonic scattering to detect subtle changes at the molecular level in liquids are conserved in ORYL's instrument while the measurement is >200x faster than the state-of-the-art. This instrument thus unlocks the use of SHS for high-throughput applications, with minimal use of compounds/chemicals while maintaining high sensitivity for subtle changes at the molecular level.

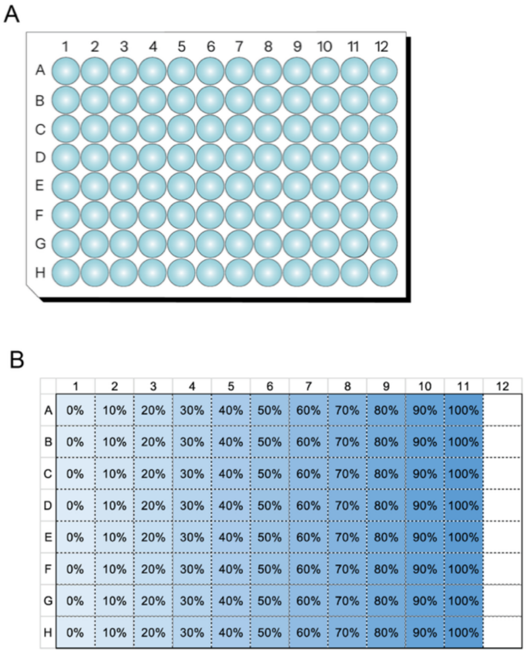
Experimental details

Materials

- H_2O: we use ultrapure water from a Milli-Q UF plus instrument (Millipore, Inc.), with an electrical resistance of 18.2 MΩ cm
- D_2O: 99.8% (16630-0250 from Acros)
- NMR tubes: cylindrical glass sample cell (4.2 mm inner diameter, LS instruments)
- Wellplates: UV-STAR® MICROPLATE, 96 WELL, HALF AREA, - 675801 (gbo.com)

Sample preparation

For the goniometric reference measurements, the inside of NMR tubes was rinsed with MilliQ water to remove surface impurities. Then 11 mixture samples of heavy water/water were prepared with increasing content of heavy water, from 0% to 100% D_2O by increment of 10%. 800 μL of mixture was pipetted into the NMR tubes before closing them with a plastic cap. Similarly, for the measurements on ORYL's F1, a 96 well plate was prepared by preparing another set of 11 mixtures with the same D2O and H2O stock, from 0% to 100% D_2O. Then 8 replicates of the series of 11 were pipetted with 100 μL total volume in each well, following the plate layout indicated in Figure 2.



	1	2	3	4	5	6	7	8	9	10	11	12
A	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	
B	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	
C	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	
D	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	
E	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	
F	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	
G	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	
H	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	

Figure 2. A. Schematic of the 96-well plate used for the measurements. B. Plate layout of the 8 replicates of the mixtures in percentage of heavy water/water content.

High-throughput well-plate reader

We used the ORYL F1 instrument developed by ORYL Photonics, adapted to measure samples in well-plates. The instrument measures standard well-plates with flat COC transparent bottom, containing the liquid samples (mixtures of H_2O/D_2O) prepared in dilution series. A measurement of one replicate of 11 samples (100 μL each) took 21.2 s while the total measurement of 8 replicates took 233 seconds (3 minutes, 53 seconds).

Goniometric setup

We used a state-of-the-art goniometric setup in a laser class 4 laboratory to measure angle-resolved second harmonic scattering, described in Ref. [2], in SSS polarization combination (perpendicular to the scattering plane). Data was measured with angle-resolved steps of 5 degrees from -40 to 40° (17 steps in total). The datapoints at 0° angle were excluded in the analysis afterwards to remove the influence of the incident beam. We used 1 second integration time, 20 measurements per angle. Each of the 11 sample mixtures required >6 minutes of measurement time (17 angular steps with 1 second integration time and N=20 datapoints). The total measurement for the 11 samples took >66 minutes, excluding the idle time when changing samples manually that adds typically >11 minutes in total.

Results: Comparison between goniometric setup and ORYL F1

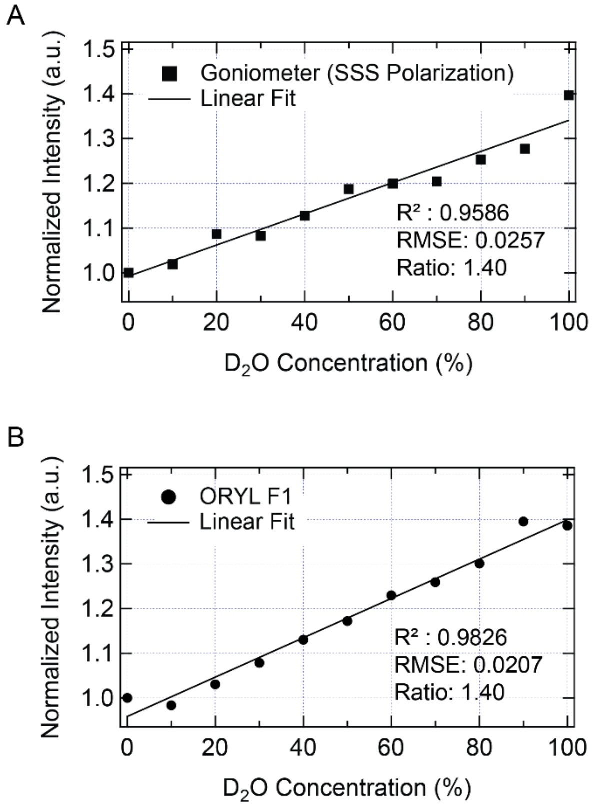


Figure 3. SHS intensity measurements of H_2O/ D_2O mixtures with A) the goniometric setup and B) with the ORYL F1 (average of 8 replicates). The intensity is normalized to the intensity of the pure water (0% D_2O concentration)

SHS intensity was measured with the goniometric setup for each sample in SSS polarization combination. The average intensity was computed from 20 datapoints at each angle, then integrated over all angles. Afterwards the integrated intensity was normalized to the value of the pure H_2O. The normalized SHS intensity for the SSS patterns as a function of increasing D_2O concentration is shown in Figure 3A (see SI for how the integrated SSS intensity was calculated).

With the ORYL F1 the SHS intensity measurement was done by averaging over 10 datapoints of 0.05 s measurement for each of the 11 D_2O/H_2O mixtures. The intensity for one set of D_2O concentration was then averaged over the 8 replicate lines, and normalized to the value of the pure H_2O. The normalized SHS intensity as a function of increasing D_2O concentration is shown in Figure 3B for comparison with the goniometric measurement.

Both figures show a linear increase of SHS intensity of around 40% when fully deuterating the water. The ratio of 1.40 (intensity of pure D_2O divided by intensity of pure H_2O) in SHS intensity matches previous studies with a goniometric setup[3]. This increase translates higher stiffness of the hydrogen bond network of D_2O that impacts the organization of the liquid at the molecular level[4].

A linear regression was performed on every series (resulting fit indicated as a line on Figure 3A and B), providing values for R^2 (the higher the better) and RMSE (the lower the better). The regression on the measurement series with the goniometric setup produces a R^2 value of 0.95 and RMSE of 0.0257, compared to 0.98 and 0.0207 with the ORYL F1.

Therefore, the ORYL F1 measures the correct intensity ratio of 1.4, while slightly beating the state-of-the-art SHS setup with respect to RMSE and R^2. This is a remarkable result given the dramatic usability, speed and sample consumption advantages of the commercial instrument.

Reproducible measurements from ORYL F1 with >200x higher throughput

To validate further the performance of the ORYL F1, we analyze the repeatability and reproducibility of the 8 replicate measurements. The normalized SHS intensity as a function of D_2O concentration for the 8 replicates and their average is shown in Figure 4. Error bars are calculated with the standard deviation (std) from the 8 replicates. All replicates show an apparent increase that follows the same trend.

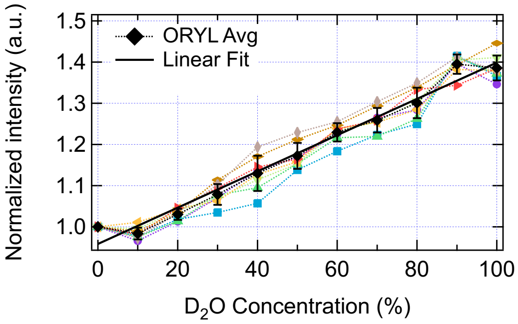


Figure 4. SHS intensity as a function of D_2O concentration for the 8 replicates and their average. The intensity is normalized to the intensity of the pure water (0% D_2O concentration).

To evaluate how the ORYL F1 can reliably distinguish D_2O concentration variations, we look at the coefficient of variation CV (defined as std/mean). The detailed values of average normalized intensity, corresponding std of the 8 replicates, and CV values are provided for each D_2O concentration in Table 1. The CV is on average 2.24% for the whole concentration series, an excellent result.

Table 1. Values of D_2O concentration, average normalized intensity, corresponding standard deviation for each of the 8 replicates, and CV values. The last line computes the average CV value for the whole concentration series excluding the 0% D_2O concentration.

D_2O Conc. (%)	ORYL F1 Avg	ORYL F1 Std	CV (%)
100	1.386	0.030	2.19
90	1.395	0.024	1.70
80	1.301	0.037	2.83
70	1.259	0.030	2.35
60	1.229	0.022	1.80
50	1.172	0.032	2.70
40	1.130	0.043	3.78
30	1.078	0.025	2.33
20	1.030	0.013	1.31
10	0.983	0.014	1.46
0	1	0	0
		Avg CV	**2.24%**

To evaluate further the reproducibility of the results between replicates, we show the normalized intensity ratios, R^2 and RMSE values for each replicate in Table 2. The ratio is consistently within a range of 1.34 and 1.45, while the R^2 and RMSE are respectively >0.922 and <0.045, highlighting the excellent reproducibility of the measurements between each replicate.

Table 2. Normalized SHS intensity ratios, R^2 and RMSE values for each of the 8 replicates.

Replicate	Ratio	R^2	RMSE
Rep1	1.3463	0.9580	0.0316
Rep2	1.3659	0.9222	0.0441
Rep3	1.3722	0.9455	0.0363
Rep4	1.4110	0.9736	0.0261
Rep5	1.3797	0.9763	0.0228
Rep6	1.3849	0.9835	0.0192
Rep7	1.4456	0.9884	0.0178
Rep8	1.3787	0.9602	0.0327

The quality of the measurement is maintained without increasing the noise compared to the goniometer. In particular RMSE value is conserved with the new instrument. The ORYL F1 achieves this high reproducibility while decreasing sample consumption and dramatically increasing the measurement throughput in comparison to the goniometer setup.

One data point in the goniometric series in Figure 3 is the result of the integration of a pattern of 16 angular datapoints, each of them being the result of 20 replicates of measurements over 1 s, hence 320 s total integration, and around 6 minutes when considering moving times. The total measurement for the 11 samples took **66 minutes** excluding the idle time when changing samples manually. On the other hand, one replicate datapoint with the ORYL F1 in Figure 4 is the result of 10 measurements of 0.05 s, hence 0.5 s total integration for a datapoint and 5.5 s for the 11 samples in the replicate. The total measurement took **21,2 s** per replicate and did not require any manual handling in between measuring samples or replicates due to the convenience of the well plate compatibility. Therefore, when considering handling times on the goniometer, the ORYL F1 is **more than 200 times faster** to measure than the state-of-the-art goniometer while preserving the quality of the data.

Conclusions

Second Harmonic Scattering is sensitive to the molecule ordering in liquids. SHS measurements of D_2O/H_2O mixtures typically exhibits a 40% higher intensity from heavy water compared to pure water that is correlated to a stiffer hydrogen bond network. We have used these mixtures to benchmark the performance of the ORYL F1 instrument against a state-of-the-art goniometric lab setup. The results show that the same measurement quality can be achieved on the F1 as in the laboratory, with the advantages of dramatically higher throughput (200x), lower sample consumption (10x), and much better usability due to the F1 being a well-plate compatible benchtop instrument, and the reference setup requiring a laser class 4 safety laboratory operating on single vials.

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Contact

ORYL Photonics SA

Route de la Corniche 5B
1066 Epalinges
Switzerland

info@orylphotonics.com

www.orylphotonics.com