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### Water enriched in the rare stable isotopes

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## Chapter 4

### **A new high-quality set of singly ( $^2\text{H}$ ) and doubly ( $^2\text{H}$ and $^{18}\text{O}$ ) stable isotope labelled reference waters for biomedical and other isotope-labelled research**

#### Abstract

This chapter describes the production and certification of two new sets of three reference waters enriched in the stable isotopes: one set singly labeled waters, only enriched in  $^2\text{H}$ , and another set of doubly Labeled Waters, enriched in both  $^2\text{H}$  and  $^{18}\text{O}$ . These reference waters have been prepared gravimetrically, and the process has led to highly accurate isotopic values for these waters. The three parent waters: natural water, pure  $^2\text{H}$  water and highly  $^{18}\text{O}$  enriched water have been thoroughly assessed for their isotopic compositions. To ensure the integrity and correctness of the gravimetric procedure, validation measurements have been carried out on the isotopic composition of the reference waters by two of our laboratories. These measurements corroborate the values obtained on the basis of gravimetric data.

This set of certified reference materials, covering enrichments in the range of 800-16,000 ‰ and 100-2,000 ‰ for  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ , respectively, is now available (called IAEA-604 to 609) and they replace (and are of much higher quality than) the exhausted reference materials IAEA-302 and IAEA-304. They will be distributed by the IAEA in 5-mL aliquots in borosilicate ampules and are valuable as reference materials for biomedical research, for e.g. body composition analyses (singly labeled water), and metabolic rate measurements (doubly labeled water). The two waters with the lowest enrichments will also be useful as anchor values for isotope measurements around the natural range. The supplies are estimated to last for over 15 years.

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## 4.1 Introduction

Analysis of the stable isotope ratios of hydrogen and oxygen has widespread applications in a variety of sciences, such as atmospheric research [1,2], hydrology [3,4], (paleo)climatology [5-8], and medicine and biology. An example of the latter is the application of stable isotopes in water as tracers to assess the water fraction of the total body mass [9-11], and the energy expenditure of humans and animals [12-14]. Both methods share their non-destructive, non-restrictive character: a small portion of water labelled with one or both stable isotopes must be administered to the person or the animal, and one (singly labelled) or two (doubly labelled) samples of one of the possible body fluids (saliva, blood, urine) must be taken. The way in which the singly labelled method establishes the total amount of body water (and thus produces valuable information about body composition) is straightforward: it is based on the dilution of the original administered isotope label by the total body water. Provided the administered amount and the enrichments of the labelled water are well known, the labelled water has been fully equilibrated through the body, and the final isotope measurement is well-calibrated, total body water can be established with high precision. The principles of the Doubly Labelled Water (DLW), invented by Lifson and colleagues in the early 1950s, are more complicated [15-17]. The DLW method uses the enrichment of the body water of a subject with known concentrations of both  $^2\text{H}$  and  $^{18}\text{O}$ , and the establishment of the turnover rates of these isotopes by taking two samples separated in time. The concentration of both  $^2\text{H}$  and  $^{18}\text{O}$  decreases by dilution through water exchange between the body and the outside world. In addition to that, the  $^{18}\text{O}$  concentration also decreases through its incorporation in  $\text{CO}_2$  produced by respiration. Therefore the decay rate for  $^{18}\text{O}$  in the body is faster than that for  $^2\text{H}$ . The difference in the decay rates of  $^2\text{H}$  and  $^{18}\text{O}$  is proportional to the production of  $\text{CO}_2$ , and is thus directly related to energy expenditure over the period of time between the two samples. The decay rate difference between  $^2\text{H}$  and  $^{18}\text{O}$  is mostly relatively small (10-15 % of the decay rates themselves). The individual rates must therefore be established accurately, which implies that laboratories should be able to accurately measure both the isotopes over several orders of magnitudes on the isotope ("delta") scale: the "initial" samples are much more isotope-enriched than the "final" samples, and natural ("background") waters need to be analyzed as well. Such a performance is only feasible when a series of reliable, well-calibrated reference waters is available.

To facilitate the DLW technique, two sets of two isotopically enriched reference materials were available through the International Atomic Energy Agency (IAEA) in Vienna, named IAEA-302 (enriched in  $^2\text{H}$ , with  $\delta^2\text{H}$  values of  $\sim 500$  ‰ and  $\sim 1,000$  ‰) and IAEA-304 (enriched in  $^{18}\text{O}$ , with  $\delta^{18}\text{O}$  values of  $\sim 250$  ‰ and  $\sim 500$  ‰), the values of which had been determined as consensus values from a ring test [18]. However, these reference waters have been exhausted for several years, while there is an ever-growing need for certified, enriched reference waters in sufficiently large quantities to serve these research fields. Therefore, we decided to produce one set of singly,  $^2\text{H}$ -enriched waters, 20 liters each, with nominal values for  $\delta^2\text{H}$  of 800 ‰, 6,000 ‰ and 16,000 ‰, and one set of doubly labelled ones, 10 liters each, with approximately the same  $\delta^2\text{H}$  values and with  $\delta^{18}\text{O}$  values of nominally 100 ‰, 750 ‰ and 2,000 ‰. For maximum accuracy and reliability of the assigned isotope values, we produced the waters using a combination of precise characterization of the three "parent" waters and gravimetric mixing.

In this chapter, we report the production and certification of these waters. In the following paragraphs we first discuss the available starting materials and the actual gravimetric mixing process. We report on our scrutiny of the parent waters: natural water (through a ring test), pure  $^2\text{H}$ -water and  $^{18}\text{O}$ -enriched water. Then, the gravimetric mixing procedure by which the new reference waters have been produced will be discussed, and how this leads to a reliable estimate of the isotope values for the reference waters. Finally, we report our best gravimetric  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values for the new reference waters, with their combined uncertainties. The whole production process has been monitored and validated using isotope measurements in two of our laboratories (The Center for Isotope Research (CIO) of the University of Groningen, and at IAEA).

As a side result, we developed, in three independent efforts, spreadsheets to accurately compute isotope values based on a combination of waters with different isotopic composition. All three give identical results. We have used these spreadsheets for our present work; they will be discussed in the following chapter, and the final version is available on our website. Important for this chapter, however, is that the calculations are based on the absolute abundance ratios for the international calibration material, the water VSMOW:  $^2\text{H}/^1\text{H} = 155.76$  ppm,  $^{18}\text{O}/^{16}\text{O} = 2005.20$  ppm, and  $^{17}\text{O}/^{16}\text{O} = 379.9$  ppm [19]. These numbers are needed to bring waters for which the isotopic abundances are stated on common grounds with those for which the isotope values have been determined based on the delta scale convention.

## 4.2 Experimental Section

### 4.2.1 Gravimetric preparation of Reference Materials

The basis of the production of the new reference materials is gravimetric mixing of a distilled natural water sample (typical  $\delta^2\text{H}=-42.7\text{‰}$  and  $\delta^{18}\text{O}=-6.3\text{‰}$ ) with isotopically pure  $^2\text{H}$  water (specified  $^2\text{H}$  fraction =99.993 %, Sigma Aldrich, Zwijndrecht, the Netherlands) and highly  $^{18}\text{O}$  enriched water (specified as  $^{18}\text{O}$  fraction =97.6 % and  $^{17}\text{O}$  fraction =0.7 %, Cortecnet, Voisins-Le-Bretonneux, France). For preparation and temporary storage of these reference waters, we used 30-liter stainless steel containers that are normally used for potable liquids (wine). These containers have lids that are part of the containers. The lids are equipped with a self-developed easy water extraction system avoiding evaporation and contamination with water vapor from the atmosphere when drawing water.

We filled each of the three containers for singly labelled waters initially with about 20 kg of demineralized local tap water (Elix 10, Millipore, Amsterdam, Netherlands) and the three other containers for doubly labelled waters with about 10 kg (for all 6 reference waters from the same single supply tank) and determined the mass of the water to a precision of better than  $\pm 1$  gram (better than 1:104) using a newly purchased precision balance (WLC 12/30/C1/R, RADWAG, Radom, Poland). The isotopically enriched parent waters,  $^2\text{H}$  and  $^{18}\text{O}$  water, were served in small vials such that the quantities (ranging from 1.5 to 56 grams) could be weighed to 0.01 mg to 0.1 mg precision (better than 1:105) with a precision analytical balance (CPA225D, Sartorius, Göttingen, Germany). Care was taken to restrict evaporative losses to the very minimum by covering the vials, and working quickly. The set of the three singly labelled waters was made by immersing the small glass vials filled with the  $^2\text{H}$  water in the corresponding three containers for thorough mixing with the demineralized waters. Subsequently, the containers were tightly capped. The three doubly labelled waters were produced by immersing simultaneously the filled small vials with the  $^2\text{H}$  and those filled with the highly enriched  $^{18}\text{O}$  water into their three containers. The all-airtight containers were stored for three weeks, during which period they were regularly moved/rolled to ensure complete mixing. Prior to mixing, we calculated the "recipes" of the reference waters (that is, the masses of the natural, the  $^2\text{H}$  and the  $^{18}\text{O}$  waters for each of them) using our spreadsheets with the specifications of the manufacturers for  $^2\text{H}$  and  $^{18}\text{O}$ . Of course, the final values differ somewhat from the nominal values we aimed for, caused by the

actual weights of the waters we put together, and the true isotope values of the waters that we determined, as described in the next paragraph.

We checked the calibration of both balances using appropriate, certified calibration weights, and established their correctness. Given the desired accuracy of the weighing process, a correction for the buoyancy effect must be applied. At 21 °C, 1013 hPa (the Groningen laboratory is at sea level) and 60 % relative humidity, the air mass is 1.19 kg/m<sup>3</sup> or 0.12 % of that of water [20]. The correction for this buoyancy effect would therefore lead to about 0.12 % higher masses than those read from the balances. However, as we are not using the masses themselves, but only mass ratios, the buoyancy correction largely cancels. The only effects that remain are caused by day-to-day variations in air pressure and temperature, and by the density differences between the normal and (heavy isotope) enriched waters we use. The former effects, pressure and temperature variation, lead to at most  $\pm 1$  % variation in the air density and thus in variations in the water mass ratios of at most  $10^{-5}$  (and likely much less, since all water weighing and mixing has been done on a per barrel basis on the same day). This is an insignificant uncertainty contribution. For weighing natural and enriched waters, however, the buoyancy effect leads to a systematic difference of slightly above  $10^{-4}$  in their mass ratios. That influence is significant, and we corrected for this effect. With densities (at 21 °C) of 998 kg/m<sup>3</sup> for natural water, 1105 kg/m<sup>3</sup> for pure <sup>2</sup>H<sub>2</sub>O, and 1106 kg/m<sup>3</sup> for our highly enriched, but not pure, <sup>18</sup>O water [21-24], we find that we overestimate the weight of <sup>2</sup>H<sub>2</sub>O by  $1.15 \times 10^{-4}$  and that of our <sup>18</sup>O water by  $1.16 \times 10^{-4}$  relative to the natural water. Therefore, we corrected the weights of <sup>2</sup>H<sub>2</sub>O and <sup>18</sup>O water by these small amounts for the calculations of the isotope values. For all other mixtures described later we also applied a buoyancy correction if significant. As it turns out, this is only the case when natural and <sup>2</sup>H- or <sup>18</sup>O-water are mixed; for mixing natural with even our highest enriched reference water of +16,000 ‰ for  $\delta^{2}\text{H}$  and +2,000 ‰ for  $\delta^{18}\text{O}$ , the buoyancy correction for the ratio is negligible, as the density of this water is only about 0.6 kg/m<sup>3</sup> higher than that of natural water [25].

#### 4.2.2 Quantification of the parent waters

##### 4.2.2.1 Characterization of Natural water

An Inter-laboratory comparison study has been performed for precise assessment of the isotope values for the natural water used for the reference waters. Five laboratories have measured the

$\delta^2\text{H}$  and  $\delta^{18}\text{O}$  of the natural water. Water was distributed in airtight 30-mL plastic bottles and securely capped to prevent any isotopic change. The reported values expressed on the VSMOW-SLAP scale of this intercomparison are shown in Table 1.

Table 1. The isotope ratio values of natural water with their stated combined uncertainties as determined by five laboratories.

Lab number	Method	$\delta^{18}\text{O}$ (‰) vs VSMOW-SLAP	$\delta^2\text{H}$ (‰) vs VSMOW-SLAP
1	Laser Spectrometry	-6.22±0.07	-42.1±0.6
2	Laser Spectrometry	-6.34±0.05	-42.7±0.3
3	Mass Spectrometry	-6.30±0.03	-42.5±0.4
4	Mass Spectrometry	-6.36±0.03	-43.3±0.5
5	Mass Spectrometry	-6.70±0.10	-42.9±0.4
Best value		-6.32±0.04	-42.7±0.4

There is one obvious outlier for  $\delta^{18}\text{O}$  in the table; averaging the others yields the best values for our natural water:  $\delta^{18}\text{O} = -6.32 \pm 0.04$  ‰ and  $\delta^2\text{H} = -42.7 \pm 0.4$  ‰, in which we estimate the combined uncertainty conservatively (all uncertainties mentioned in this work are 1- $\sigma$ ).

#### 4.2.2.2 Characterization of pure $^2\text{H}$ water

The highly  $^2\text{H}$ -enriched water used for preparation of our reference materials had a high  $^2\text{H}$  purity grade of 99.993 %, certified by the supplier (Sigma-Aldrich) for this specific batch through  $^1\text{H}$ -NMR spectroscopy. The results generated by a dilution testing method in our laboratory for determination of the  $^2\text{H}$  purity corroborated this value, but with much lower precision. Therefore, we relied on the stated purity, but used a conservative uncertainty estimate of  $\pm 0.005$  % in this value.

We made a set of three singly labelled waters (now called IAEA-604, 605, 606 in a sequence of increasing  $\delta^2\text{H}$  values) by gravimetric mixing of this deuterated water with the natural water (information on the masses are shown in Table 2). Because this deuterated water is also used for

a set of doubly labelled waters, its oxygen isotopic composition is of importance, too. There was no information provided on the oxygen content of the deuterated water by the supplier. However,  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$  measurements on the new singly labelled reference waters indicated that both  $^{17}\text{O}$  and  $^{18}\text{O}$  in the deuterated water were enriched, but to a substantially lower level than the  $^2\text{H}$  (as can be expected). Therefore, to create a mixture with moderate enrichment of these O-isotopes, we made a 1:17 dilution of the deuterated water with natural water (the same natural water as described above). Still, the measurement method for  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$  of this mixture has to cope with extremely enriched levels of  $^2\text{H}$ , such that all the normally very rare "clumped" species  $^2\text{H}^2\text{H}^{16}\text{O}$ ,  $^1\text{H}^2\text{H}^{17}\text{O}$  and  $^1\text{H}^2\text{H}^{18}\text{O}$ , and even  $^2\text{H}^2\text{H}^{18}\text{O}$  and  $^2\text{H}^2\text{H}^{17}\text{O}$  cannot be neglected. A laser spectrometric measurement was therefore deemed not suitable. Instead, we decided to use the classical  $\text{H}_2\text{O}-\text{CO}_2$  equilibrium technique [26], followed by Isotope Ratio Mass Spectrometry (IRMS) analysis of the equilibrated  $\text{CO}_2$ , which is not hampered by the presence of high concentrations of  $^2\text{H}$ . Normally, this method only reveals  $\delta^{18}\text{O}$  (and  $\delta^{13}\text{C}$ , assuming the  $\delta^{17}\text{O}-\delta^{18}\text{O}$  relation for natural waters [27]), but when the  $\delta^{13}\text{C}$  of the  $\text{CO}_2$  gas used for equilibration is known (and applying a small correction for the fractionation of the dissolved  $\text{CO}_2$ ) one can alternatively interpret the IRMS results for masses 45 and 46 in terms of  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$ . At our CIO Groningen lab, we have been performing this alternative method for decades for our DLW measurements. Some years ago, Elsig et al [28] have driven the method to perfection. The precision of  $\delta^{17}\text{O}$ , however, is limited, as it has to be deduced from about 6 % of the mass-45 signal (the other 94 % is due to  $^{13}\text{CO}_2$ ). Whereas Elsig et al. [28] reach a combined uncertainty of  $\sim 0.1$  ‰, we estimate the combined uncertainty with our standard equipment to be 0.4 ‰ for natural samples.

We performed equilibrium measurements on this 1:17 diluted  $^2\text{H}$ -water six times (on different days), together with measurements on the three prepared singly labelled waters. To find the best estimates for the  $^{18}\text{O}$  and  $^{17}\text{O}$  isotopic abundances of the deuterated water, we considered its abundances as fit parameters to minimize the sum of differences between the measured and calculated  $\delta$ -values for our measurements. Both the measurement results and the fit values are shown in Table 2. The fit values are calculated using the spreadsheets we developed, using the masses of the constituents (buoyancy ratio corrected for the deuterated water) and the appropriate values for their isotope content.



Table 2. Assessment of  $^{18}\text{O}$  and  $^{17}\text{O}$  in  $^2\text{H}$ -enriched water using IRMS; combined uncertainties in the measured values are given in brackets. The uncertainties in the masses vary from  $\pm 0.00002$  g to  $\pm 0.00010$  g for the gram amounts, and are  $\pm 0.7$  g for the 20 kg amounts of the natural water.

Sample	Mass of $^2\text{H}$ enriched water (grams, buoyancy ratio corrected)	Mass of natural water (grams)	Measured $\delta^{18}\text{O}$ (‰) <i>and average</i>	Fitted $\delta^{18}\text{O}$ (‰)	Measured $\delta^{17}\text{O}$ (‰) <i>and average</i>	Fitted $\delta^{17}\text{O}$ (‰)
1:17 dilution of enriched $^2\text{H}$ water	1.10100	18.83035	169.4 (0.3) 169.0 (0.3) 169.1 (0.3) 169.1 (0.3) 169.2 (0.3) 169.0 (0.3) <i>169.1</i>	169.1	62.4 (0.5) 62.0 (0.5) 62.4 (0.5) 62.5 (0.5) 62.5 (0.5) 62.2 (0.5) <i>62.3</i>	62.3
IAEA-604	3.21228	22000.2	-5.95 (0.06) -5.91 (0.06) -5.91 (0.06) -5.92	-5.86	-3.0 (0.4) -3.0 (0.4) -3.4 (0.4) -3.2	-3.2
IAEA-605	20.9764	20041.4	-3.04 (0.06) -3.12 (0.06) -3.09 (0.06) -3.08	-3.02	-2.1 (0.4) -2.1 (0.4) -2.1 (0.4) -2.1	-2.1
IAEA-606	56.1803	20219.0	2.39 (0.06) 2.39 (0.06) 2.41 (0.06) 2.40	2.43	0.3 (0.4) -0.5 (0.4) -0.3 (0.4) -0.2	-0.1

The result for the  $^{18}\text{O}$  abundance in the deuterated water is  $0.8998\pm 0.0010\%$ , about a factor of 4.5 higher than natural. The  $^{17}\text{O}$  abundance is  $0.0874\pm 0.0004\%$ , about 2.3 times the natural abundance. The  $^{17}\text{O}$  abundance in the waters that we use for mixing is important not only for its own sake, but also because it influences the other values. The obtained results are necessary information for the calculation of  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$  of the doubly labelled waters.

The mentioned uncertainties in the estimated abundances are the result of the described fit procedure. The results for the 1:17 water obviously dominate the process, so their combined uncertainties mainly determine the uncertainty in the results. It is worth noting that the spread of the individual measurements, which is a measure for the repeatability, is much lower than the individual combined uncertainties suggest. However, the most important uncertainty source, which all individual measurements have in common, is caused by the scale normalization uncertainty; for these values far out of the VSMOW-SLAP range, this uncertainty contribution dominates.

#### 4.2.2.3 Characterization of the highly $^{18}\text{O}$ -enriched water

Contrary to the deuterated water, the  $^{18}\text{O}$ -enriched water was not certified with satisfactory accuracy for our goal. This is caused by the fact that a purity of virtually 100 % cannot be reached in the thermo-diffusion process by which  $^{18}\text{O}$  is enriched. Furthermore, the other rare isotope of oxygen,  $^{17}\text{O}$ , gets enriched as well. The purchased commercial  $^{18}\text{O}$  enriched water for preparation of doubly labelled waters had been specified to an enrichment level of  $^{18}\text{O}=97.6\%$  and  $^{17}\text{O}=0.7\%$  by the supplier (in fact the company sold the water with the guarantee of  $^{18}\text{O} > 95\%$  enrichment). For our purposes, an independent determination of the  $^{18}\text{O}$  enrichment level is thus essential. It can be undertaken through gravimetric dilution of the  $^{18}\text{O}$ -enriched water by natural water that is depleted in  $^2\text{H}$  and  $^{18}\text{O}$  (and  $^{17}\text{O}$ ) such that the end product is in its isotopic composition close to the international calibration water VSMOW. However, starting directly from for example 1 gram of  $^{18}\text{O}$ -enriched water (a lower quantity prohibits accurate enough weighing) is not feasible due to the large amount of natural depleted water needed to reach the isotopic composition of VSMOW. Therefore the most economical way of performing this test is to first gravimetrically produce our three doubly labelled waters, as described above, and use aliquots of those for further dilution. This is the procedure we followed. As explained above, for the calculation of the necessary quantities of labelled and natural water, we initially used the

specifications by the manufacturer. This way, we produced the three doubly labelled reference waters, now called IAEA-607, 608 and 609 in a sequence of increasing  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  values. Table 3 provides information on the mass contents of the doubly labelled waters.

Table 3. Masses of the parent waters used for preparation of IAEA-607, 608, and 609 with nominal  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$  values. The uncertainties in the gram amount masses are as specified in Table 2, and are  $\pm 0.3$  g for the 10 kg amounts of the natural water.

Samples	Nominal $\delta^2\text{H}$ (‰)	Nominal $\delta^{18}\text{O}$ (‰)	Mass of contents (grams)		
			Natural water	pure $^2\text{H}$ water	$^{18}\text{O}$ enriched water
IAEA-607	800	100	10012.6	1.46262	2.42248
IAEA-608	6000	750	10006.5	10.48975	17.08363
IAEA-609	16000	2000	10024.7	27.9682	45.4676

We took samples of these waters and diluted them with a natural local reference water (OC1), a water from Antarctica we obtained from the EPICA community (European Project for Ice Coring in Antarctica [29], O. Catani, personal communication). The  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  determined for OC1 over the period of 8 years of its availability in our lab are  $-424.5 \pm 0.6$  ‰ and  $-53.99 \pm 0.06$  ‰, respectively, relatively close to the second scale calibration material SLAP. We mixed the waters with OC1 in such quantities that the  $\delta^{18}\text{O}$  values of the mixtures were close to VSMOW in isotopic composition. We made a total of eleven of such mixtures. These samples were subsequently measured by IRMS using the equilibration method described above. In a similar procedure as described above, we used the  $^{18}\text{O}$  and  $^{17}\text{O}$  concentration of the original  $^{18}\text{O}$  parent water as fit parameters, and we optimized the correspondence between the calculated  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$  values for the mixtures and the actual measurements. Two of the eleven mixtures showed significant differences between the fit values and the actual measurements, which we attributed to errors in the mixing process. They were excluded from the fit process. The results for all samples are shown in Table 4 (the two erroneous mixtures appear on the last two rows in italics).

Table 4. Measured and fitted  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$  values for eleven independent mixtures of IAEA-607, IAEA-608, and IAEA-609 and our natural local standard water OC1 (depleted in  $^2\text{H}$ ,  $^{17}\text{O}$  and  $^{18}\text{O}$ ) to assess the  $^{18}\text{O}$  and  $^{17}\text{O}$  abundance in the  $^{18}\text{O}$  enriched water. Uncertainties in the masses are as given before, the individual combined uncertainties for  $\delta^{18}\text{O}$  measurements are  $\pm 0.06$  ‰, and for  $\delta^{17}\text{O}$   $\pm 0.4$  ‰. The two lowest rows show the results of two mixtures that we discarded from the fitting process.

Sample	Mass of Doubly labelled water (grams)	Mass of OC1 (grams)	Average of Measured $\delta^{18}\text{O}$ (‰) (# of mm)	Fitted $\delta^{18}\text{O}$ (‰)	Average of Measured $\delta^{17}\text{O}$ (‰)	Fitted $\delta^{17}\text{O}$ (‰)
IAEA-609+ GS-OC1	1.0196	40.2591	-4.41 (4)	-4.39	-24.9	-25.0
IAEA-609+ GS-OC1	1.01189	37.0013	-0.85 (1)	-0.53	-24.9	-24.8
IAEA-609+ GS-OC1	1.00114	37.6470	-2.05 (2)	-1.98	-25.0	-25.3
IAEA-609+ GS-OC1	1.00418	37.6502	-1.84 (2)	-1.83	-24.7	-25.2
IAEA-608+ GS-OC1	1.00330	13.99809	-1.20 (1)	-1.54	-23.9	-22.6
IAEA-608+ GS-OC1	0.99974	14.14832	-1.91 (2)	-1.92	-24.0	-24.0
IAEA-608+ GS-OC1	0.99970	14.20044	-2.06 (2)	-2.10	-24.1	-24.0
IAEA-607+ GS-OC1	1.00150	1.91209	-1.36 (2)	-1.41	-17.3	-17.7
IAEA-607+ GS-OC1	0.70052	1.34035	-1.44 (2)	-1.48	-17.5	-17.7
IAEA-609+ GS-OC1	<i>1.01742</i>	<i>39.1358</i>	<i>-1.68 (3)</i>	<i>-3.10</i>	<i>-25.1</i>	<i>-25.0</i>
IAEA-609+ GS-OC1	<i>1.02555</i>	<i>37.9946</i>	<i>-1.92 (4)</i>	<i>-1.21</i>	<i>-25.2</i>	<i>-24.8</i>

The combined uncertainties in the measured values are  $\pm 0.06$  ‰ for  $\delta^{18}\text{O}$  and  $\pm 0.4$  ‰ for  $\delta^{17}\text{O}$ . The fitted  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$  values in the Table above correspond to abundances of  $96.05 \pm 0.12$  ‰ for  $^{18}\text{O}$  and  $1.176 \pm 0.020$  ‰ for  $^{17}\text{O}$ . The uncertainties are based on the fit process, but also on the changes in the results that the fit process would give if we would include one or both of the excluded samples. Clearly, the specified enrichments of  $^{18}\text{O}=97.6$  ‰ and  $^{17}\text{O}=0.7$  ‰ by the supplier are not very accurate.

The hydrogen isotopic composition of the  $^{18}\text{O}$ -enriched water needs to be measured too. The high-temperature pyrolysis-IRMS technique is insensitive to the oxygen isotopic composition, and thus we used our experimental set-up (chromium reduction oven (Pyr-OH, Eurovector, Milan, Italy) and a continuous flow IRMS (Isoprime, Manchester, UK)) to perform  $\delta^2\text{H}$

measurements on mixtures with the  $^{18}\text{O}$ -enriched water. Since the  $^2\text{H}$  isotope is obviously far less enriched than the  $^{18}\text{O}$  one, we could not use the mixtures of Table 4 for a reliable  $\delta^2\text{H}$  determination of the original  $^{18}\text{O}$  water. Therefore, we made a diluted sample of the  $^{18}\text{O}$  water itself, such that its  $^2\text{H}$  abundance leads to a significant signal. Table 5 shows the experimental data on this dilution.

Table 5. The  $\delta^2\text{H}$  values of the diluted  $^{18}\text{O}$ -enriched water with our reference water OC1 ( $\delta^2\text{H}=-424.5 \pm 0.6 \text{ ‰}$  and  $\delta^{18}\text{O}=-53.99 \pm 0.06 \text{ ‰}$ ), as measured with our chromium reduction IRMS system. The uncertainties in the masses are as given before.

sample	Mass of $^{18}\text{O}$ enriched water (grams, buoyancy ratio corrected)	Mass of OC1 (grams)	Measured $\delta^2\text{H}$ (‰)
dilution of enriched $^{18}\text{O}$ water	0.50149	40.0050	-302.2±0.5

Fitting the  $\delta^2\text{H}$  of this dilution to the measured one leads to a  $^2\text{H}$  abundance of  $0.1791 \pm 0.0014 \text{ ‰}$  in the  $^{18}\text{O}$  water, more than an order of magnitude above the natural abundance.

It is clear that the "other" side of commercially available, highly enriched  $^2\text{H}$  and  $^{18}\text{O}$  water is substantially enriched too (although often declared "renormalized" by the suppliers). This additional information on the enriched waters is crucial for producing reference waters with accurate and reliable delta values. Still, as shown below, the largest uncertainty contribution is caused by the uncertainty in the  $^{18}\text{O}$  concentration of the  $^{18}\text{O}$  water itself.

### 4.3 Results and combined uncertainties for the new reference waters

Based on the above determinations of the full isotopic composition of the two enriched parent waters, the (buoyancy ratio corrected) weights of the three constituents of the mixtures, and the delta values of the natural water, we could calculate the final delta values of the six new reference waters. The values, along with their (1- $\sigma$ ) combined uncertainties, and also the equivalent isotopic abundances are given in Table 6.

For the calculation of the final combined uncertainties, we treated the various uncertainty sources per reference water as independent. In principle there are eight (for the singly labelled waters) or twelve (for the doubly labelled waters) uncertainty sources: the weights of the waters used for mixing (two for the deuterated reference waters, three for the doubly labelled ones) and the three isotopic compositions of each of the two/three source waters. However, only a limited number of them played a significant role. For the  $\delta^2\text{H}$  values, up to four uncertainty sources were significant (though not for all six waters). Figure 1 shows these sources and their relative importance in the combined uncertainty. For the reference waters with low enrichment the uncertainty in  $\delta^2\text{H}$  of the natural water is the dominant source, and this gradually changes with the higher enrichments, where the uncertainty of the  $^2\text{H}$  abundance in the deuterated water and, somewhat surprisingly, the uncertainty in the weight of the natural water take over. For  $\delta^{18}\text{O}$ , the situation is much simpler: the uncertainty in the new reference waters is entirely due to the uncertainty in the  $^{18}\text{O}$  abundance of the  $^{18}\text{O}$ -water. For the waters with (close to) natural  $\delta^{18}\text{O}$  values the uncertainty in  $\delta^{18}\text{O}$  of the natural water dominates. For  $\delta^{17}\text{O}$ , the situation is completely analogous to that of  $\delta^{18}\text{O}$ , only with larger relative uncertainties.

Table 6. Isotopic compositions of the new reference waters, expressed both in delta values and isotopic abundances, with their combined uncertainties in brackets.

Reference	$\delta$ -values and combined uncertainties(‰)			Absolute Isotopic abundances (ppm)		
	$\delta^2\text{H}$	$\delta^{18}\text{O}$	$\delta^{17}\text{O}$	$^2\text{H}$	$^{18}\text{O}$	$^{17}\text{O}$
IAEA-604	799.9 (0.4)	-5.86 (0.04)	-3.2 (0.4)	280.28 (0.06)	1988.73 (0.08)	377.80 (0.15)
IAEA-605	5997.9 (0.5)	-3.02 (0.04)	-2.1 (0.4)	1088.80 (0.08)	1994.40 (0.08)	378.20 (0.15)
IAEA-606	15993.6 (1.0)	2.43 (0.04)	-0.1 (0.4)	2639.93 (0.15)	2005.28 (0.08)	378.97 (0.15)
IAEA-607	802.4 (0.4)	99.02 (0.13)	3.6 (0.4)	280.67 (0.06)	2198.07 (0.25)	380.29 (0.16)
IAEA-608	6014.3 (0.5)	736.4 (0.9)	45.7 (0.8)	1091.35 (0.08)	3468.4 (1.7)	395.7 (0.3)
IAEA-609	16036.4 (1.0)	1963.7 (2.2)	126.6 (2.2)	2646.57 (0.16)	5905 (4)	425.3 (0.8)

The combined uncertainties in Table 6 are valid for the bulk reference waters. In the end, these waters will be distributed in 5-mL aliquots. The final combined uncertainty for the end-users thus has to include the possible heterogeneity introduced during the glass sealing of ampoules. As in previous operations, this “between-bottle” heterogeneity will be thoroughly tested by

random selection of 6 out of batches of 500 ampoules for each reference material. These will be analyzed under repeatability conditions for possible isotopic differences. The process will be repeated at different measurement days to calculate the variability between ampoules, the repeatability for each ampoule and the reproducibility of measurements after several days. From these data the maximal residual uncertainty component attributable to heterogeneity will be calculated. If this uncertainty is not negligible compared to the combined uncertainties of Table 6, the total end-user combined uncertainty will be increased. The final end-user combined uncertainties will be provided in the reference sheets that accompany the sets of ampoules that will be distributed.

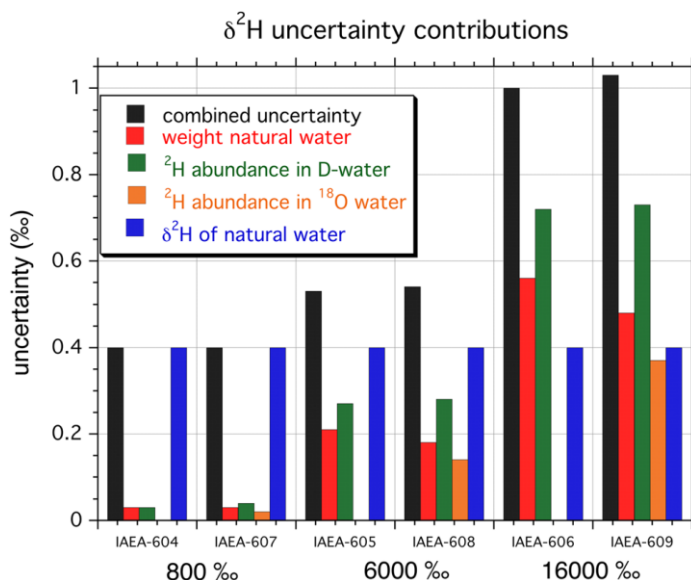


Figure 1. The significant contributions to the combined uncertainties in  $\delta^2\text{H}$  of the six reference waters. For the low enrichments, the uncertainty in the  $\delta^2\text{H}$  of the natural water is the dominant uncertainty source, but for the higher enrichments several uncertainty contributions start playing a role. For the highest enrichment, the uncertainty in the  $^2\text{H}$  abundance in the deuterated water is the largest contributor.

#### 4.4 Consequences of uncertainties in the calibration materials VSMOW and SLAP

The final values in Table 6, and their combined uncertainties, rely on the measured and accepted values for the isotope ratios of VSMOW [30-32] (which are inherited by its successor material VSMOW2) that we have used in our analysis without attributing an uncertainty to them. In that way, we present values that are consistent with all other isotope measurements presented on the

delta scales. The second calibration water for the  $^2\text{H}$  and  $^{18}\text{O}$  isotope scales is SLAP (with its successor SLAP2), and the difference between VSMOW and SLAP has been defined by consensus to be  $\delta^2\text{H}$  (SLAP w.r.t. VSMOW) = -428 ‰,  $\delta^{18}\text{O}$  (SLAP w.r.t. VSMOW) = -55.5 ‰. In addition, we used in our calculations the value of  $\delta^{17}\text{O}$  of SLAP:  $\delta^{17}\text{O}$  (SLAP w.r.t. VSMOW) = -29.70 ‰, which is consistent with the  $\delta^{17}\text{O}$ - $\delta^{18}\text{O}$  relation as found by Meijer and Li [27], and which has been recently recommended by Schoenemann et al. [33]. Whereas the values for VSMOW directly influence the relation between the highly enriched  $^2\text{H}$ - and  $^{18}\text{O}$  waters on the one hand, and the natural water on the other, the values for SLAP influence the interpretation of our dilution measurements (Tables 2, 4 and 5), as the delta values of the OC1 reference water depend on them in a nearly 1:1 relation (as OC1 is close to SLAP in its isotopic composition). The original publications, cited by Gonfiantini [19], give uncertainties in the abundances (or rather isotope ratios) for both VSMOW and SLAP that are not negligible compared to the combined uncertainties we give in Table 6. The way these uncertainties influence our assigned values to the new reference waters is quite different for  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ , and we will treat these cases separately.

For  $\delta^2\text{H}$ , the influence of uncertainty in the abundance in VSMOW is straightforward: the relative uncertainty of  $\pm 3.2 \times 10^{-4}$  in the  $^2\text{H}$  abundance of VSMOW ( $155.74 \pm 0.05$  ppm) directly translates into the same relative error in the  $^2\text{H}$  abundances of the reference waters. Except for the lowest enrichments of IAEA-604 and IAEA-607 (for which this amounts to  $\pm 0.27$  ‰), these uncertainties, translated into  $\delta^2\text{H}$  values, are considerably larger than the combined uncertainties in table 6:  $\pm 2.0$  ‰ for IAEA-605 and IAEA-608, and even  $\pm 5$  ‰ for IAEA-606 and IAEA-609. For  $\delta^{18}\text{O}$ , the situation is more complex: contrary to the  $\delta^2\text{H}$  case, we could not rely on the enrichment of the  $^{18}\text{O}$  water, but instead determined this ourselves using dilution with natural  $^{18}\text{O}$ -depleted water. In this case, both the uncertainty in the  $^{18}\text{O}$  abundance in VSMOW ( $2000.4 \pm 0.5$  ppm, or a relative uncertainty of  $2.5 \times 10^{-4}$ ), and the delta value of SLAP with respect to VSMOW play a role.

The effect of a change in the  $^{18}\text{O}$  abundance of VSMOW is remarkable: it does not change the assignment of  $\delta^{18}\text{O}$  values to the reference waters at all. Instead, the fit procedure used for the characterization of the highly  $^{18}\text{O}$  enriched water (described above) results in a relative change in the  $^{18}\text{O}$  abundance of the  $^{18}\text{O}$  water (and, in second order, of the  $^{18}\text{O}$  abundance of the deuterated water as well) that is the same as the relative change in VSMOW, such that the fit values are



identical to the ones in Table 4. Given the uncertainties in the  $^{18}\text{O}$  abundance values for the  $^{18}\text{O}$  water reported above, an extra relative uncertainty of  $\pm 2.5 \times 10^{-4}$  is negligible.

The influence of the  $\delta^{18}\text{O}$  value for SLAP, however, is considerable. Contrary to the  $^2\text{H}$  situation, there is no independent measurement of the  $^{18}\text{O}$  abundance of SLAP reported in the literature. The value of SLAP,  $\delta^{18}\text{O} = -55.5 \text{ ‰}$  with respect to VSMOW, is a consensus value based on a large intercomparison study in the 1980s [19]. Looking at the distribution of measurement results at that time, however, it is likely that its actual value is somewhat more negative. Also our own long-term experience (described in [34]) shows that its value is rather  $\sim -55.8 \text{ ‰}$ . If we would use that value to normalize our  $\delta^{18}\text{O}$  scale for all measurements involved, it would mean all our  $\delta^{18}\text{O}$  measurements, including those for our natural parent water and, most of all, the local reference water OC1, would change by a factor of  $55.8/55.5 \approx 1.0054$ . Consequence of this change is that now both the  $^{18}\text{O}$  abundance in the  $^{18}\text{O}$  water and the  $\delta^{18}\text{O}$  values of the new reference waters would increase by that factor, in other words undergo a relative change of  $5.4 \times 10^{-3}$ . The  $^{18}\text{O}$  abundance in the  $^{18}\text{O}$  water would then be 96.63 % instead of 96.05 %, and IEAE-609 would have a  $\delta^{18}\text{O}$  of 1974.4 ‰ instead of 1963.7 ‰. These differences, however, cannot be interpreted simply as "uncertainty". Our impression is (loosely based on talks about this subject with other expert colleagues, latest at the IAEA Technical Meeting on Stable Isotope Reference Materials, Vienna, 1-5 September 2014) that it is generally agreed that the value for  $\delta^{18}\text{O}$  of SLAP is actually more negative than  $-55.5 \text{ ‰}$ :  $-55.80 \pm 0.10 \text{ ‰}$  might be a reasonable estimate for both its value and its uncertainty. This would imply relative uncertainties of (only)  $1.8 \times 10^{-3}$  in the  $\delta^{18}\text{O}$  values of our reference waters, still almost twice as large as the combined uncertainties in Table 6.

Thus, although two of the three uncertainty sources discussed above are significant or even major compared to the uncertainties given in Table 6, for most applications of these reference waters they do not need to be taken into account. If researchers (using isotope dilution or DLW techniques) use the same values for the abundances in VSMOW when converting abundances into delta values and vice versa, and use the VSMOW-SLAP scale in their experiments with the recommended values for SLAP, the whole system is consistent, and the uncertainties treated here cancel. Only in special applications, like for instance the certification of the  $^{18}\text{O}$  abundance in  $^{18}\text{O}$  water, these uncertainties need to be taken into account.

If the isotope community should one day decide to change the recommended value for  $\delta^{18}\text{O}$  of SLAP (or of SLAP2 as the de facto now available calibration water), it is straightforward to recalculate the values for the reference waters that belong to such a new value.

#### **4.5 Experimental validation of the reference waters**

Provided all procedures went as expected, the gravimetric values for these reference waters are superior to directly measured ones in terms of combined uncertainty. However, to check if all procedures indeed went right, an experimental verification of the values is most useful. Therefore we analyzed the prepared reference waters IAEA 604-609 in our two laboratories, CIO and IAEA. The IAEA performed independent measurements using a Cavity Ring Down Spectrometer (L2130-i, Picarro, Santa Clara, Ca, U.S.A.), both on the reference waters themselves and on dilutions with a selection of three internal standards. The measurements have been calibrated using the normal laboratory procedures for the natural range, leading to a considerable extrapolation for those direct measurements of the reference waters. This leads to higher combined uncertainties for the higher enrichments.

At CIO, the waters were measured with three different techniques: pyrolysis-IRMS for simultaneous measurement of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ ,  $\text{H}_2\text{O}-\text{CO}_2$  equilibrium-IRMS for measurement of  $\delta^{17}\text{O}$  and  $\delta^{18}\text{O}$ , and laser spectrometry on water vapor for  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ , using a commercial prototype ('SARA', Floralis, Grenoble, France) of an instrument based on a variant of CRDS [35-37].

The pyrolysis measurements have been calibrated using our local set of doubly labelled waters. These have been made in the same fashion as the present set of reference waters, only at least an order of magnitude less accurate. For the equilibrium system we used our normal natural reference waters for calibration, thus leading to a considerable extrapolation (as in the IAEA case). For SARA, we decided to calibrate the measurements using the lowest and highest enrichments of the new reference waters themselves (IAEA-604 and IAEA 609), so the measurements using SARA are more of a consistency check for the whole series.

Figure 2 shows the differences between the four sets of results and the values in Table 6. The error bars of the data points are the best estimates of the combined uncertainties in the measurements, whereas the gray zones around zero represent the combined uncertainty in the assigned values for our new reference waters.

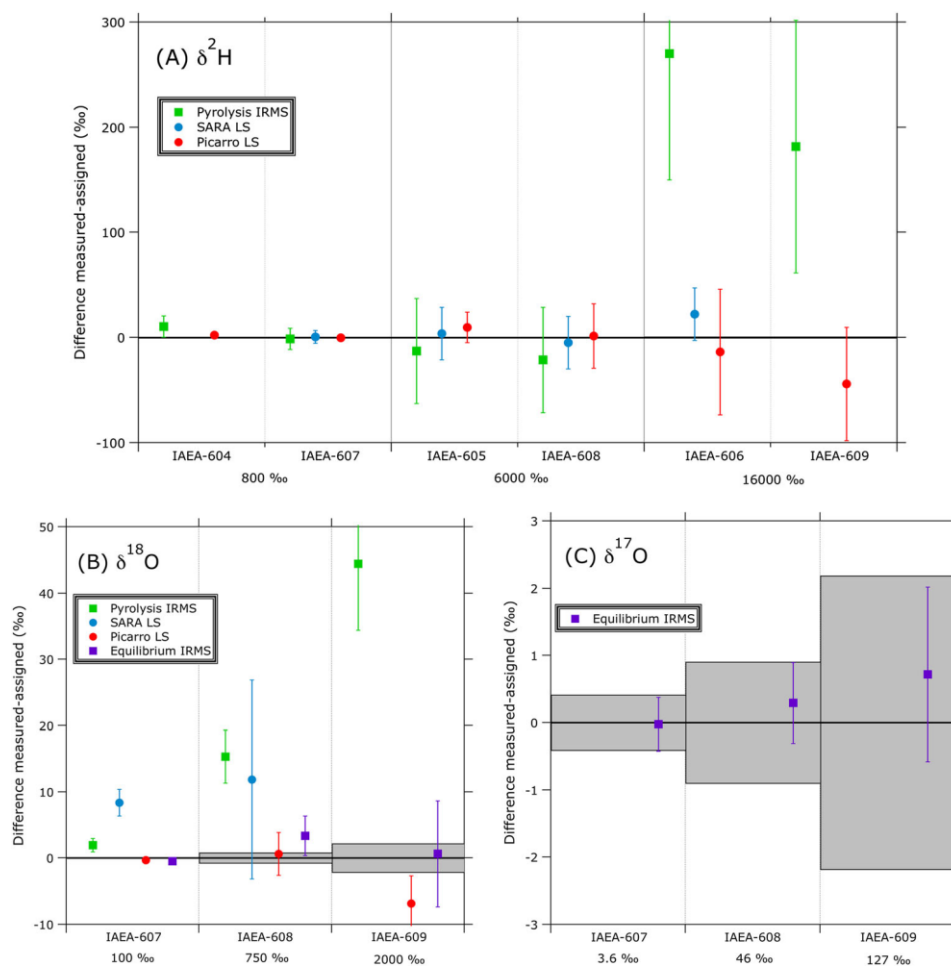


Figure 2. The differences between measurements using four different techniques and the assigned values (see Table 6) of the new reference waters. The gray zones around zero represent the combined uncertainty in the assigned values of the reference waters. The pyrolysis based results for both  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  (green squares in both (A) and (B)) show systematic deviations, due to deviation in the local DLW reference waters these results have been calibrated with. The other techniques show reasonable to good agreements. For all measurement techniques the new reference waters represent a huge improvement in terms of precision and accuracy for both  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ , as the width of the area around zero is negligible ( $\delta^2\text{H}$ , (A)) or small ( $\delta^{18}\text{O}$ , (B)) compared to the spread of all methods. For  $\delta^{17}\text{O}$  (C) this is not the case, but the reference waters have not been made for the purpose of  $\delta^{17}\text{O}$ -calibration.

The measurements of  $\delta^2\text{H}$  (Figure 2A) show in general good agreement; only the pyrolysis-based results for the highest enrichments show deviations (of  $\sim 1.5$  to  $2\text{-}\sigma$ ). This is an indication that the

local DLW reference waters in use at CIO are not reliable (anymore) in this high enrichment range. For all measurement techniques the new reference waters will be a huge improvement in terms of precision and accuracy: the widths of the gray zones around zero are negligible compared to the spread of the data points for all measurement methods.

Figure 2B shows the  $\delta^{18}\text{O}$  results for the three doubly labelled reference waters IAEA-607, 608 and 609. The pyrolysis measurements deviate even more significantly than in the  $\delta^2\text{H}$  case, another indication that the DLW reference waters might have lost their reliability (or that some other problem occurred). The other measurement methods show reasonable to good results. Although the gain in accuracy is not so impressive here as in the  $\delta^2\text{H}$  case, the improvement is still very significant.

Figure 2C shows the results for  $\delta^{17}\text{O}$ . Although the  $\delta^{17}\text{O}$  results for these reference waters are meant as indicative only (and to make clear that the  $\delta^{17}\text{O}$ - $\delta^{18}\text{O}$  relation for these waters is not at all natural, which might influence the results of some measurement techniques), the agreement between the equilibrium measurements and the assigned values of the reference waters is very good indeed. Based on this figure (and thus on the equilibrium measurements) one can conclude that the combined uncertainties for the  $\delta^{17}\text{O}$  values may well be lowered by a factor of two.

All in all, based on the results shown in Figure 2 we can be confident that the reference waters have indeed been prepared according to plan and that the values presented in Table 6 are reliable.

#### 4.6 Conclusions

We have succeeded in making a series of new, isotope-enriched, reference waters, in sizeable quantities, with well-characterized isotope ratios using gravimetric mixing. In the process it appeared to be crucial to characterize the original parent waters as carefully as possible. It turned out that (1) the abundances of  $^{18}\text{O}$  and  $^{17}\text{O}$  for the highly enriched  $^{18}\text{O}$  water as provided by the supplier were not accurate (enough) and (2) the isotopic abundances of the non-specified side of such highly enriched waters (that is,  $^2\text{H}$  in  $^{18}\text{O}$  water and  $^{17,18}\text{O}$  in  $^2\text{H}$  water) are substantially enriched as well. We determined the composition of these waters using dilutions with a natural, water depleted in  $^2\text{H}$  and  $^{18}\text{O}$  (and  $^{17}\text{O}$ ), for which the isotope delta values were well known, and this appeared to be a reliable process, leading to accurate numbers for the isotope abundances. The work also highlighted the importance of maintaining consensus values for the isotopic abundances of the international calibration water VSMOW, and the definition of the difference

between VSMOW and the second calibration material SLAP. If, however, these consensus values should change because of new findings, the isotopic values for the reference waters can be recalculated with such new consensus values in a straightforward manner. The close coupling between the abundance of  $^{18}\text{O}$  in the  $^{18}\text{O}$  water, and the isotopic value for SLAP with respect to VSMOW hint towards a new way to establish this value experimentally, something that we plan to pursue in the near future. For the successor materials of VSMOW and SLAP, the two nowadays available calibration materials VSMOW2 and SLAP2, no isotopic abundance measurements exist (only a thorough intercomparison between them and VSMOW and SLAP, described in the reference sheet available on the IAEA reference materials website).

The validation measurements made clear how valuable these new reference waters will be for fields in which isotopically enriched waters need to be measured, such as the "doubly labelled water" technique for energy expenditure, in which the enrichment difference between  $^2\text{H}$  and  $^{18}\text{O}$  contains the signal. The improvement in accuracy will be impressive for  $\delta^2\text{H}$ , and very significant for  $\delta^{18}\text{O}$ . This will make these reference waters, especially those with the lowest enrichments, even useful for isotope measurements in the natural range, for which they can serve as high anchor point. As an example of the latter, IAEA-604 is already in use in a ring test of  $\delta^2\text{H}$  for solid materials (A. Schimmelmann, at the IAEA Technical Meeting on Stable Isotope Reference Materials, Vienna, 1-5 September 2014), which will better connect the  $\delta^2\text{H}$  scale for solid materials to VSMOW, as was done earlier for  $\delta^{18}\text{O}$  [38]. The  $\delta^{17}\text{O}$  of the reference waters is provided as well, not so much to let these waters also serve as  $\delta^{17}\text{O}$  reference, but rather to specify the  $\delta^{17}\text{O}$ - $\delta^{18}\text{O}$  relation of these waters, which is far from the natural one. This might slightly influence  $\delta^{18}\text{O}$  measurements of these waters in IRMS techniques where an isotopologue mass interference plays a role, such as in the water- $\text{CO}_2$  equilibration process ( $^{12}\text{C}^{18}\text{O}^{16}\text{O}$  with  $^{13}\text{C}^{17}\text{O}^{16}\text{O}$ ) or in pyrolysis ( $^{12}\text{C}^{18}\text{O}$  with  $^{13}\text{C}^{17}\text{O}$ ).

The reference waters IAEA-604 through -609 are now available through the usual IAEA channels for isotope reference materials. They are distributed in 5-mL aliquots in borosilicate ampules.

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