

Enrichment of H₂¹⁷O from Tap Water, Characterization of the Enriched Water, and Properties of Several ¹⁷O-Labeled Compounds

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A low-abundance form of water, H₂¹⁷O, was enriched from 0.04% to ~90% by slow evaporation and fractional distillation of tap water. The density and refractive index for H₂¹⁷O are reported. Gas chromatography–mass spectrometry (GC-MS) of ¹⁶O- and ¹⁷O-1-hexanols and their trimethyl silyl ethers and of ¹⁶O- and ¹⁷O-hexamethyl disiloxanes was used to determine the percentage of ¹⁷O enrichment in the H₂¹⁷O. Furthermore, the chemical shifts of labeled and nonlabeled water dissolved in CDCl₃ differed sufficiently that we could verify the enrichment of H₂¹⁷O. ¹⁷O hexanol was synthesized by the reaction of iodohexane with Na¹⁷OH. ¹⁷O-Labeled trimethylsilanol and ¹⁷O-labeled hexamethyldisiloxane were prepared by the reaction of H₂¹⁷O with bis(trimethylsilyl)trifluoroacetamide (BSTFA). To generate standards for ¹⁷O NMR, H₂¹⁷O₂, and ¹⁷O camphor were prepared. H₂¹⁷O was electrolyzed to form ¹⁷O-labeled hydrogen peroxide which was quantified using two colorimetric assays. ¹⁷O-Labeled camphor was prepared by exchanging the ketone oxygen of camphor using H₂¹⁷O. The ¹⁷O-labeled compounds were characterized using ¹⁷O, ¹H, and ¹³C NMR and GC-MS. While we were characterizing the labeled camphor, we also detected an unexpected oxygen exchange reaction of primary alcohols, catalyzed by electrophilic ketones such as camphor. The reaction is a displacement of the alcohol OH group by water. This is an example of the usefulness of ¹⁷O NMR in the study of a reaction mechanism that has not been noticed previously.

Dioxygen, O₂, is central in many processes of life, such as photosynthesis and respiration. Many of the enzymatic mechanisms in anabolic and catabolic processes require O₂ and generate oxygen-containing intermediates or products. Examples of enzymes that require oxygen atoms at some point in their catalytic cycles, either as O₂, superoxide, H₂O₂, or water, include three important groups. First are the heme-containing enzymes, such as cytochromes P450,^{1,2} cyclooxygen-

ase,^{3,4} dioxygenases,^{5,6} or NO synthases.⁷ Second are flavin-containing oxidoreductases,⁸ such as putrescine oxidase or Baeyer–Villigerases,⁹ and third are nonheme iron-containing enzymes, such as fatty acid desaturases.¹⁰ Despite its high (1.229 V) oxidation potential, dioxygen is not very reactive under standard conditions in living organisms because O₂ is in the triplet ground state, whereas most metabolites are in a singlet ground state. The superoxide ion, O₂^{•−}, is formed through a one-electron reduction of O₂. Upon a further reduction and protonation, superoxide forms hydrogen peroxide, H₂O₂, a mild oxidant or reductant.¹¹ Further reduction and protonation yields water, the least reactive oxygen species in this chain. The enzymes listed above exploit the differential reactivities in this chain of oxygen species, from O₂ to water in their catalysis. The reduction of O₂ to water (O₂ + 4e[−] + 4H⁺ → 2 H₂O, ΔG = −474 kJ/mol)¹² is highly exothermic, and cytochromes P450 or fatty acid desaturases exploit this property, utilizing the free energy released in the reduction of O₂ to activate hydrocarbon C–H bonds. These enzymes accomplish this feat of thermodynamic coupling by coordinating with O₂ and reducing it in a stepwise manner. Some reactive oxygen species are sometimes released prematurely from these enzymes, leading to oxidative stress in the cell.¹³ It is, therefore, of interest to study how the various oxygen species interact with enzymes.

Oxygen has three stable isotopes: ¹⁶O (abundance 99.759%), ¹⁷O (0.037%) and ¹⁸O (0.204%). ¹⁶O and ¹⁸O have a nuclear spin (I) of zero whereas ¹⁷O has I = 5/2 which makes it detectable by NMR spectroscopy.¹⁴ Pure H₂¹⁷O is the commonly accepted reference standard¹⁵ for the chemical shifts in ¹⁷O NMR. ¹⁷O

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chemical shifts span a range -30 to $+600$ ppm¹⁶ which makes distinguishing functional groups containing oxygen (bonded to carbon, nitrogen, or sulfur) relatively straightforward, despite the low abundance and high cost of ^{17}O . Due to the lack of interferences, enzymatic samples can, in principle, be studied directly by ^{17}O NMR without the need to remove the protein, making it a practical tool for mechanistic studies. ^{17}O NMR studies have enormous applications in chemistry and biology. For example, Gullion et al.¹⁷ have reported the determination of secondary structures in polyamides by ^{13}C – ^{17}O READPOM NMR. ^{17}O NMR is also used in imaging analysis to determine the cerebral metabolic rate of oxygen in rats.¹⁸

Commercially available H_2^{17}O is prohibitively expensive (1 g of 90% enriched H_2^{17}O costs $>\$2000$), and in order to facilitate and expand the use of ^{17}O -labeling studies in enzymatic reactions, there is a need for an economical method by which researchers can enrich ^{17}O from water and characterize the isotopic enrichment in a simple and reliable way. In this paper we describe an inexpensive method for enriching both H_2^{17}O and H_2^{18}O in tap water using slow evaporation followed by fractional distillation. We also report simple procedures to determine the percentage of isotopic enrichment of ^{17}O -labeled water using gas chromatography–mass spectroscopy (GC-MS) of 1-hexanol and hexamethyldisiloxane (HMDS) synthesized from H_2^{17}O and deionized water. The fractional distillation method reported here is based on the differences in the volatility of the three forms of water that vary in their oxygen isotope.^{19–21}

In our research group we use the isotope-enriched water for the study of enzymatic reactions of P450_{cam}, a camphor hydroxylase. We therefore also describe the preparation of ^{17}O -labeled hydrogen peroxide by the electrolysis of H_2^{17}O , synthesis of ^{17}O -labeled camphor and report the ^1H , ^{13}C , and ^{17}O NMR data for ^{17}O 1-hexanol, ^{17}O camphor, ^{17}O trimethylsilanol, and ^{17}O hexamethyldisiloxane. Furthermore, while studying the labeled camphor in H_2^{17}O , we detected an unusual ^{17}O exchange into the ethanol that was used to deliver camphor into the water. This is an important example that illustrates how ^{17}O NMR can provide insight into reactions that may otherwise have gone unnoticed.

EXPERIMENTAL SECTION

Enrichment of H_2^{17}O from Tap Water. Tap water (~ 1 – 2 L) was placed in a shallow black bowl and left to evaporate slowly at room temperature on a windowsill. When the water in the bowl reached ca. 20–50 mL, it was collected into a measuring cup and briefly boiled in a microwave (~ 30 s high power) to kill any bacteria that may have accumulated. The sterilized enriched water was stored in a glass jar with a tight lid. This process was repeated until more than 1 L of enriched water had been accumulated. The

water was filtered through fluted filter paper (Whatman Cat. No.1001-070), to remove any particulate matter, and ~ 500 mL of this was placed in a 1 L round-bottom flask, fitted with two condensers (Supporting Information Figure S1), for fractional distillation.

Fractional Distillation of the Enriched Water. The vertical condenser was packed with glass wool and was not cooled with running water. The tilted condenser at the top had cold water running to condense the distillate. The system was attached to a single-neck still head that could be rotated easily in order to allow various fractions of water to be collected without interrupting the distillation. The distillation source flask was heated with a mantle connected to a Variac (setting: 50). To ensure good fractionation, it was important that the water was not heated too quickly.

The boiling point was monitored using a thermometer at the top of the fractionation column (Supporting Information Figure S1), and several fractions with different boiling points were collected. Temperatures given are not corrected. For reference, the SFU Burnaby campus lies 370 m above sea level, and the boiling point of tap water registers at 97°C in our apparatus at this location. Fractions having boiling points of 98.5°C (10 mL \times 6) and 99°C (10 mL) were collected.

Preparation of Hydrogen Peroxide by the Electrolysis of Water. Hydrogen peroxide is a redox-active compound that is most commonly encountered as an oxidant.²² Industrially, anthraquinone is hydrogenated to form anthrahydroquinone which is further oxygenated to form hydrogen peroxide.²³ Other methods for synthesizing H_2O_2 include hydrolysis of peracids (e.g., peracetic acid),²⁴ and enzymatic hydrolysis of phosphatidic acid to glycerol-3-phosphate (G3P), which is then oxidized by G3P oxidase to hydrogen peroxide.²⁵ Catalytic methods of production using palladium membranes²⁶ and zirconium catalysts²⁷ have also been reported. Several electrolytic methods for hydrogen peroxide generation have been reported in the literature, including a fuel-cell method,^{28–30} electrolysis of water using a carbon cathode, and a RuO_2 -based titanium anode,³¹ using a solid-polymer electrolyte³² or using a proton-exchange membrane.³³ We required a method that uses H_2O , and so we selected electrolysis.

In our method, the electrolysis of 5 mL of H_2^{17}O buffered to pH 7.7 using 50 mM phosphate buffer (made from 50 mM KH_2PO_4 and 50 mM K_2HPO_4) with 150 mM KCl was carried out using a copper cathode and a graphite anode. The electrodes were connected to a Biorad Power Pac 1000 and a

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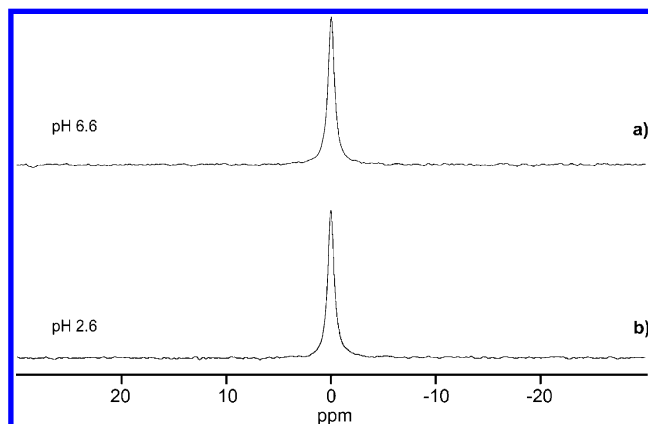
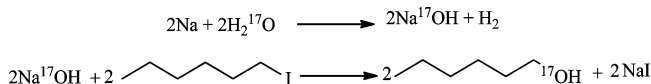


Figure 1. ^{17}O NMR spectrum of water samples: (a) pH 6.6, (b) pH 2.6.

Scheme 1. Formation of ^{17}O -Labeled Hexanol by a S_{N}^2 Reaction



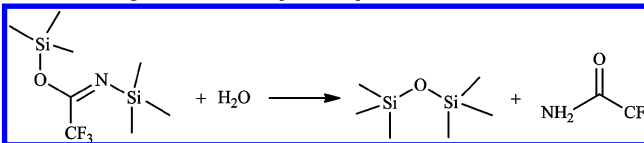
constant voltage of 5 V was applied for one hour. The reaction was monitored by observing the redox properties of H_2O_2 in two different reactions (quantitation details of H_2O_2 in the Supporting Information).

^{17}O NMR of Water. The proton decoupled ^{17}O NMR of the water sample (pH 6.6) was obtained with CDCl_3 lock in a coaxial capillary, recycle delay 0.2 s and the chemical shift was set to 0 ppm. (Figure 1a). Another H_2^{17}O sample containing buffer (pH 2.6) was also run in the same fashion to see if there is any variation in the chemical shift with pH. (Figure 1b). NMR acquisition conditions are included in the experimental details of the Supporting Information.

Synthesis of Labeled 1-Hexanol and Hexamethyldisiloxane. The ^{17}O -labeled water could not be run directly on a gas chromatograph–mass spectrometer because the fused-silica capillary column would have been damaged by the water. We chose to prepare labeled 1-hexanol because smaller alcohols are hard to detect by GC-MS (weak molecular ion peak and high volatility) and higher alcohols give more complex fragmentation patterns. ^{17}O -Labeled hexanol was synthesized by reacting the ^{17}O -enriched water with sodium metal, followed by addition of 1-iodohexane. Briefly, 200 μL (0.2 mmol) of ^{17}O water was reacted with 3 mg (0.13 mmol) of sodium metal (Scheme 1). When the metal had all reacted, 1 equiv of 1-iodohexane (10 μL was dissolved in 100 μL of acetone) was added, and the reaction mixture was stirred at room temperature for 3 h. The reaction was monitored every 30 min by GC-MS, and after 3 h, complete depletion of 1-iodohexane was observed.

A solution (1 μL diluted 1000-fold with distilled hexane) of the 1-hexanol was injected into a Varian 3800 GC, equipped with a 30 m SPB-5 column (i.d. = 0.25 mm, 0.25 μm film thickness, Supelco) interfaced with a Varian Saturn 2000 ion trap mass spectrometer. The trimethylsilyl ether of 1-hexanol was prepared by reacting 0.5 μL of 1-hexanol in a small ampule with 4 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma) for 40 min

Scheme 2. Hydrolysis Reaction of BSTFA To Form Hexamethyldisiloxane (HMDS)



at RT, and the reaction mixture was diluted with hexane (1000 \times) for the injection of 1 μL on the GC-MS. (The column oven settings are included in the experimental details of the Supporting Information).

To form hexamethyldisiloxane, HMDS (or bis-trimethylsilyl oxide) (Scheme 2), 0.5 μL of deionized water and H_2^{17}O were each treated with 5 μL of BSTFA for 40 min at 60 $^\circ\text{C}$. For HMDS GC-MS analysis, ion storage (SIS mode) was used, and m/z 135–150 amu was scanned. For ^{17}O NMR studies, 300 μL of BSTFA was treated with 25 μL of H_2^{17}O in a small ampule and left overnight at RT. About 300 μL of pentanol was added the next day, the organic extract was concentrated at RT, and 450 μL of CDCl_3 was added. In our NMR studies, ^{17}O -labeled trimethylsilanol, ^{17}O -labeled hexamethyldisiloxane (HMDS), and ^{17}O -labeled trifluoroacetamide (reaction byproduct) were detected.

Preparation of ^{17}O Camphor. Camphor (7 mg) was dissolved in 0.5 mL of CDCl_3 and added to 50 μL of H_2^{17}O buffer (50 mM phosphate, pH 7.7) in an NMR tube (diameter: 5 mm). The mixture was left to react at room temperature overnight and analyzed directly by ^{17}O NMR the next day. After NMR, a sample was extracted and checked by EI GC-MS (The GC-MS data of ^{17}O -labeled camphor is included in the Supporting Information).

RESULTS AND DISCUSSION

Density and Refractive Index. The density and refractive index experimental details are included in the Supporting Information. The density of deionized water was found to be $0.9986 \pm 0.0006 \text{ g/cm}^3$ and of H_2^{17}O fraction 11 (99%) 1.0026 ± 0.0010 at 21 $^\circ\text{C}$. This increase in density for H_2^{17}O is significant ($P = 0.014$, t test, five replicates) and expected from the observation that H_2^{18}O (99 atom $^{18}\text{O}\%$) has a reported density of 1.11 g/mL at 20 $^\circ\text{C}$.³⁴

The refractometer was calibrated with ethanol whose refractive index was found to be 1.3605, as reported in the literature.³⁵ For deionized water, the refractive index was measured to be 1.3321, and for H_2^{17}O , it was 1.3318. The literature value for H_2^{16}O is reported to be 1.3330.³⁶ The refractive index of H_2^{17}O has not been reported previously according to our knowledge.

Determination of the Percentage of H_2^{17}O in the Fractions from Distillation. We prepared two compounds to assess the percentage of H_2^{17}O in the fractions obtained from distillation: 1-hexanol and hexamethyldisiloxane (HMDS). The percentage of labeling was calculated using the integrated peak areas of the molecular ion (M^+ , for 1-hexanol) or a prominent fragment ion ($\text{M} - 16$, for HMDS) of

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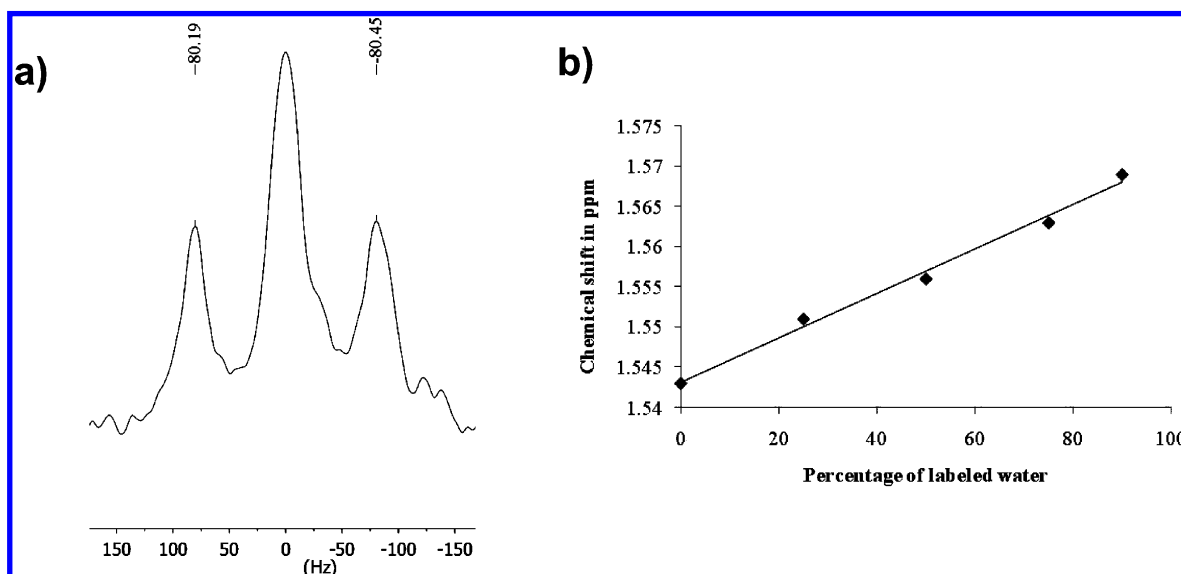


Figure 6. (a) ^{17}O NMR spectrum of 1 μL of 90% enriched water dissolved in CD_3CN (540 μL) acquired at 67.8 MHz without ^1H decoupling using 5 mm TBO probe, spectral width 800 ppm, 18935 scans, 0.2 s recycle delay, $T = 298\text{ K}$. (b) Chemical shifts of varying proportions of ^{17}O and ^{16}O water in CDCl_3 .

Chart 2

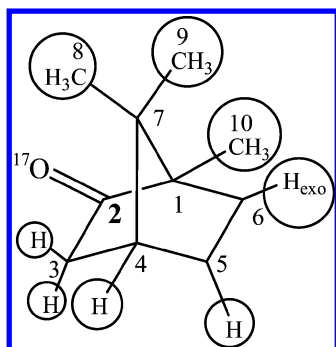


Table 2. The Effect of ^{17}O Shielding Cone on the ^{13}C and ^1H Chemical Shifts in Camphor

assignment ^a	$\Delta\delta^b\ ^{13}\text{C}$ (ppm)	$\Delta\delta^b\ ^1\text{H}$ (ppm)
1	+0.01	—
2	-0.02	—
3	+0.02	exo ($\sim+0.01$) endo ($\sim+0.01$)
4	+0.02	$\sim+0.01$
5	+0.02	$\sim+0.01$
6	+0.02	$\sim+0.01$
7	+0.02	—
8	+0.02	$\sim+0.01$
9	+0.02	$\sim+0.01$
10	+0.02	$\sim+0.01$

^a Refers to the position of carbon or hydrogen atom as numbered in Chart 2. ^b Chemical shift differences observed in the ^{13}C NMR and ^1H NMR spectra for labeled and unlabeled camphor. $\Delta\delta < 0$ indicates that the signal is shielded in labeled relative to nonlabeled camphor while $\Delta\delta > 0$ means the signal is deshielded.

The nonlabeled (mainly ^{16}O) hexanol (Sigma) derivatized by BSTFA had a retention time of 10.32 min, and ^{17}O -labeled hexanol derivatized by BSTFA had a longer retention time 10.515 min. (Supporting Information Figure S17). The ^{17}O HMDS had a retention time of 7.010 min and the ^{16}O HMDS

had a retention time of 6.865 min. (Supporting Information Figure S18). Thus, for both TMS derivatives, the retention time of the labeled compound was longer than that of the ^{16}O compounds. In the case of 1-hexanol, the isotope fractionation was sufficient to get baseline separation of the labeled and nonlabeled pair.

GC-MS Fragmentation Pattern Analysis. The nonlabeled 1-hexyl trimethylsilyl (TMS) ether showed an $M - 1$ ion at m/z 173 in its mass spectrum (Supporting Information Figure S17) whereas ^{17}O -labeled 1-hexyl-TMS ether showed the molecular ion M^+ at m/z 175. Presumably an isotope effect in the ^{17}O -labeled compound prevented the loss of a hydrogen atom (Scheme 3), as seen abundantly for the nonlabeled compound. HMDS fragmented by the loss of methane to fragment ions m/z 146 in the case of deionized water (H_2^{16}O), m/z 147 in the case of H_2^{17}O and m/z 148 in the case of H_2^{18}O (Scheme 3). The mass spectrum of the TMS ether of H_2^{17}O was (EI): m/z (% of base peak) 147 (100), 145 (12.5), 135 (25), 134 (45), 131 (35) (Supporting Information Figure S18).

The GC-MS isotope fractionation and fragmentation analysis of nonlabeled and ^{17}O labeled camphors, MS-MS data of nonlabeled and ^{17}O labeled camphors, and ^{16}O - and ^{17}O -1-hexanols is included in the Supporting Information.

4. CONCLUSIONS

An inexpensive, straightforward method for enriching ^{17}O -labeled water from tap water and the subsequent preparation of ^{17}O -labeled hydrogen peroxide from electrolysis of H_2^{17}O are described in this paper. The fractional distillation method for enrichment of H_2^{17}O reported here can greatly enrich the percentage of H_2^{17}O or H_2^{18}O which are both useful for isotope studies. From approximately 500 mL of 40-fold enriched water, about 90 mL of H_2^{17}O was obtained. The most practical method for determining the enrichment was found to be the reaction of the H_2^{17}O with BSTFA to yield

hexamethyldisiloxane which was quantified by GC-MS analysis. Five other ^{17}O -labeled compounds were also prepared from the ^{17}O -labeled water (sodium hydroxide, 1-hexanol, hydrogen peroxide, trimethylsilanol, and camphor) and characterized by NMR and GC-MS. This illustrates the power of ^{17}O NMR in the detection of the reactions of O-containing functional groups. Finally, an unexpected exchange reaction of primary alcohol moieties with water, facilitated by ketones, was detected by ^{17}O NMR.

SUPPORTING INFORMATION AVAILABLE

The experimental details, ^1H , ^{13}C NMR, and the mass spectra of camphor, hexanol, and HMDS. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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SUPPORTING INFORMATION

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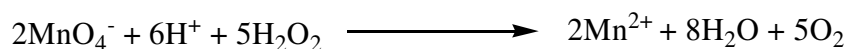
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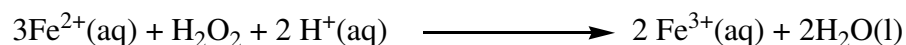
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Figure S9. ¹³ C{ ¹ H} NMR spectrum of hexamethyl siloxane and trimethyl silanol in CDCl ₃ . ---	S-17

4. Quantitation of hydrogen peroxide. Hydrogen peroxide was detected using two colorimetric assays ^{2,3}: the first tests the reducing ability of H₂O₂ and the second one tests the oxidising ability. To test the reducing ability of the H₂O₂ solution, 100 µL of the electrolysed solution was treated with 200 µL of 3% w/v trichloroacetic acid (TCA) and left at 4 °C for 20 minutes. Further treatment with 10 mM KMnO₄ resulted in the decolorisation of the permanganate.



To test for the oxidising abilities of the hydrogen peroxide, 500 µL of the electrolysed water was treated with 1000 µL of 3% w/v trichloroacetic acid (TCA) and left at 4 °C for 20 minutes. Further treatment with 250 µL of ferrous ammonium sulphate (Fe(NH₄)₂SO₄) and 125 µL of potassium thiocyanate (KSCN) resulted in the formation of a reddish brown complex (Fe(SCN)₃):



The absorbance of the Fe³⁺ (as Fe(SCN)₃ complex) was monitored at 480 nm on a UV-Visible spectrophotometer (Hach DR/4000U). A calibration curve for hydrogen peroxide concentrations was prepared using a series of solutions of known concentrations. The concentration of hydrogen peroxide obtained by electrolysis was found to be 0.1 mM using both methods.

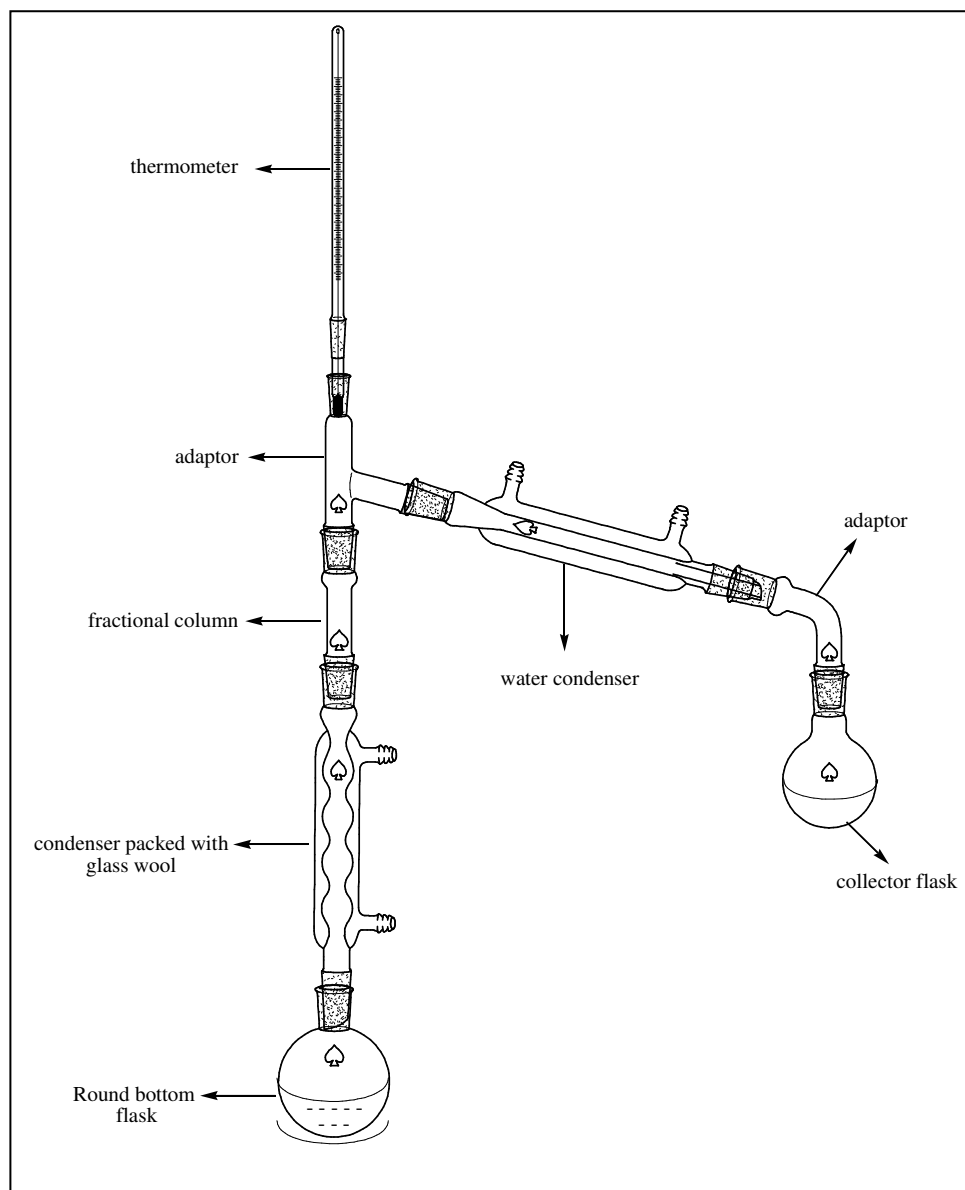


Figure S1. Enrichment of H_2^{17}O from tap water by fractional distillation.