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A protocol for the synthesis of [^{35}S]-labeled 3-dimethylsulfoniopropionate and dimethylsulfide from L-methionine for use in biogeochemical studies

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Abbreviations: dimethylsulfide (DMS), 3-dimethylsulfoniopropionate (DMSP), methylmercaptopropionate (MMPA), 3-methylthiopropionate (3-MTP), Ultra-pure deionized water (DI), polypropylene (PP)

Highlights:
- [$_{35}$S]-DMSP can be chemically synthesized from commercially-available materials
- The specific activity and yield are high enough for applications in biogeochemistry
- The protocol has had restricted use published over the past 20 years
- It could be extended to other radiolabels, depending on the chosen application

1. Abstract

Radiotracers are highly sensitive tools for quantifying the rates of important biogeochemical processes and the fates of specific atoms and/or compounds within major global elemental cycles, especially those that are requisite for life. Important radiolabeled organosulfur compounds, like dimethylsulfide (DMS) and its precursor 3-dimethylsulfoniopropionate (DMSP), are not commercially available, but their well-documented use has been key in furthering our understanding of the marine sulfur cycle. [^{35}S]-DMSP obtained by chemical synthesis has been used extensively in radiotracer studies involving DMS and DMSP, but its synthesis has been restricted to 2 research groups. Presented here is a protocol for the chemical synthesis of [^{35}S]-DMSP from [^{35}S]-L-methionine, though the method could be used for other radiolabels (e.g. [^{14}C], [^{3}H]). The synthesis consists of 2 reaction steps, (1) the sequential oxidative deamination and decarboxylation of [^{35}S]-L-methionine to [^{35}S]-3-methylmercaptopropionate and (2) the methylation of [^{35}S]-methylmercaptopropionate to yield the product [^{35}S]-DMSP. The product is purified by liquid chromatography and two cation-resin exchanges. Average final [^{35}S]-DMSP yield was 5.34% (n=16; range: 1.26% to 14.84%, excluding failures), although updated instrumentation could likely improve final yields. The objective of this work is to standardize the synthesis of [^{35}S]-DMSP to widen its availability and use among the community and hence facilitate increased understanding of the reduced sulfur and carbon cycles.

2. Introduction

Sulfur is a required element for life. It is present in many biomolecules (e.g., amino acids, proteins, polysaccharides, lipids) and can be used as an electron donor for carbon fixation by certain microbes (Hu et al., 2018; Taylor and Visscher, 1996). Of the estimated 34 Tg of sulfur emitted to the atmosphere each year from natural sources, more than half (21 Tg y^{-1}) originates in the ocean, primarily in the form of the volatile organosulfur gas dimethylsulfide (i.e. DMS; Lee et al., 2011; Brimblecombe 2014; Lana et al. 2011). DMS, and other sulfur gases, form sulfate aerosols in the atmosphere and are known to have a cooling effect on the climate (Charlson et al., 1987). As such, it is vital to understand the cycling of both the marine and global sulfur cycles to better model the sources and sinks of these climatically active compounds that link the marine environment with the atmosphere.

3-dimethylsulfoniopropionate (DMSP), besides being the precursor to DMS, is a critically important compound in the marine environment. DMSP accounts for up to 11% of cellular carbon in individual phytoplankton cells, and total global DMSP production accounts for 3-10% of total carbon fixation within the marine environment (Stefels et al. 2007; Galí et al. 2015). This single compound can also fulfill up to 13% of carbon and 100% of sulfur demands for marine microbes (Kiene and Linn 2000a; Levine et al. 2016). DMSP is a widespread source of sulfur and carbon, preferred because the sulfur moiety is reduced and thus requires less energy to assimilate over more oxidized forms. Furthermore, DMSP can serve a variety of other cellular functions in producers and consumers, including as an antioxidant, a cryoprotectant, an energy overflow mechanism, and a grazing deterrent (Sunda et al., 2002; Stefels, 2000). Consumption rates of the dissolved DMSP pool by marine microbes are rapid, with turnover times as

low as hours (Kiene and Linn, 2000b; Ledyard and Dacey 1996). As a result, surface concentrations of dissolved DMSP are typically low (<10 nM), even in blooms of high-producing phytoplankton (Kiene et al., 2019; Motard-Côté et al., 2016; Kiene and Linn, 2000a). These conditions (low DMSP stock, rapid turnover) pose challenges to studying the cycling of DMS and DMSP in the marine environment.

Synthetic radiotracers are highly sensitive tools that enable tracing the fate of specific atoms in molecules involved in complex biochemical and biogeochemical processes (Cresswell et al., 2020). Radiotracer methods facilitate direct quantification of rates associated with these processes, through the addition of low concentrations of compounds of interest without significantly altering their natural abundance and, as a result, their kinetics. As such, their use in biogeochemistry has a long and extensive history: common examples include [^{14}C]-$NaHCO_3$ to estimate primary production rates (Nielsen, 1952), [^{3}H]-leucine and [^{3}H]-thymidine to quantify bacterial growth rates and production estimates (Smith and Azam, 1992), and ^{32}Si for biogenic silica production rates (Tréguer et al., 1991). [^{35}S]-labelled compounds, including sulfate, DMSP and methionine, have been used to quantify the assimilation of [^{35}S] into the protein matter of marine bacteria (Cuhel et al., 1982; Samo et al., 2014, Kiene and Linn, 2000a). The synthesis of the radioactive sulfur isotope [^{35}S] was first documented by Andersen (1936), and the first applications were in describing chemical reactions (Voge, 1939; Voge and Libby, 1937). The isotope undergoes β^--decay (0.167 meV) and is naturally present in trace quantities. With the longest half-life of the sulfur isotopes (~87.4 d vs. ≤ 2.9 h for all others), it is the most useful for applications of radiotracer methods to determine the fate of S in molecules of interest (Brimblecombe, 2014).

The use of [^{35}S]-labelled organic sulfur compounds is key to understanding the biogeochemical cycling of these compounds in the marine environment, particularly those of global importance in the sulfur cycle, such as DMSP and DMS. However, radiolabeled versions of these organosulfur compounds are not commercially available, and thus their use in biogeochemical studies has been restricted. Initially, plants (i.e. *Wollastonia bifloral*) and algal cultures (i.e. *Platymonas subcordiformi*) were used to synthesize [^{35}S]-DMSP from [^{35}S]-L-methionine, followed by isolation and purification (Hanson et al., 1994; Kiene et al., 1998). However, these approaches typically had low yields with low specific activities (e.g. 0.037 Ci mmol^{-1}; Kiene et al., 1998) for the time and monetary investment involved. We present here a protocol for the chemical synthesis of [^{35}S]-DMSP and [^{35}S]-DMS from [^{35}S]-L-methionine. Leveraging methods pioneered in the early 2000's, this protocol has been repeatedly successful in producing purified, high specific activity [^{35}S]-DMSP (>1000 Ci mmol^{-1}; e.g. Motard-Côté et al., 2016; Lavoie et al., 2018), although it was never published in full detail. The protocol was devised and refined to generate a product which has sufficient yield to be valuable for scientific applications in the field of marine biogeochemistry, such as estimating turnover and production rates of methylated sulfur containing compounds and tracing the fate of sulfur from DMSP into transformation products. This protocol can also be applied to commercially available methionine labeled with other radioisotopes (e.g. [^{14}C], [^{3}H]) on atoms of interest, depending on the desired application. Commercially available L-[methyl-[^{3}H]]-methionine, for example, could be used to synthesize L-[methyl-[^{3}H]]-DMSP to investigate the fate of methyl groups from DMSP. Moreover, synthesized radiolabeled DMSP stocks can be chemically and quantitatively cleaved

under basic conditions to produce radiolabeled DMS stocks for similar investigations into the sulfur cycle (White, 1982). Expanding the use and applicability of this protocol beyond will further our understanding of the marine sulfur cycle through the lens of this globally-important organosulfur compound.

3. Methods

3.1 Reaction Principles

The synthesis of DMSP from L-methionine involves two main reactions: an enzyme-mediated simultaneous oxidation/decarboxylation of L-methionine to yield methylmercaptopropionate (MMPA; also known as 3-methylthiopropionate (3-MTP)), followed by a methylation of MMPA to yield DMSP. An additional step can yield DMS (discussed below). In the first reaction step, the enzyme L-amino acid oxidase transforms L-methionine into the intermediate α-keto 4-methylthio-2-oxobutyrate, which is further decarboxylated to MMPA (Figure 1A; Castellano and Molinier-Frenkel, 2017; Bunton, 1949). After this reaction, the MMPA is protonated with HCl and extracted using diethyl ether. It is subsequently deprotonated under basic conditions and back-extracted from the diethyl ether. In the second reaction, MMPA is methylated with methanol as the carbon donor under highly acidic conditions to produce DMSP (Figure 1B; Lavine et al., 1954), as well as other side products. If the desired product is DMS, the reaction can stop here, unpurified; otherwise, purification steps need to be conducted to isolate the DMSP. DMS stocks can be prepared by taking aliquots of either purified or impure

DMSP stocks and cleaving to DMS and acrylate using base (e.g. 5N NaOH, see sections *3.4.4* and *3.4.10*; White, 1982).

3.2 Instrumentation

All glassware was acid-washed in 10% HCl (stock: 36.5 to 38.0%; Fisher Chemical) and muffled for 4 h at 450°C (Thermo Fisher Scientific BF51800 Series Box Furnace) to remove remaining organic residues. A Supelco Visiprep D-L solid-phase extraction manifold (Cat. No. 5-7044) and disposable solid phase extraction columns were used to purify the product using a cation-exchange resin. Reaction and evaporation steps (sections *3.4.1-3.4.4, 3.4.6*, and *3.4.9*) were carried out on a standard heat block (VWR Scientific) in a radiation certified fume hood. Teflon tubing connected to a Teflon straw was used to pipe in N_2 gas for evaporations; the height of the straw was adjusted using a standard ring stand and clamp. A Shimadzu LC-10AD chromatograph with a Whatman Partisil 10 SCX was used for product separation, though other cation exchange columns could be substituted. A PerkinElmer Tri-Carb B3110 TR scintillation counter was used to quantify radioactivity and reaction yield. Standard safety and handling protocols were followed per institutional guidelines: labware and instrumentation were most often dedicated to the radioactive work, and those needed in non-radioactive applications were flushed or cleaned thoroughly and verified as non-radioactive before further use.

3.3 Reagents and Resin Cleaning

All reagents were of analytical grade or higher unless specified otherwise. Pure stocks of [^{35}S]-L-methionine (Revvity; 5 mCi in 10 mM β-mercaptoethanol; specific activity >1000 Ci mmol^{-1}) and L-amino acid oxidase (Sigma-Aldrich; from *Crotalus adamanteus*) were newly acquired for each synthesis. The [^{35}S]-L-methionine should be ordered as close to the synthesis start date as possible due to the duration of the synthesis (~2 weeks) and half-life (~87 days) of the isotope. Standard safety protocols were followed regarding the storage and disposal of radioactive waste, per institutional guidelines. Ultra-pure deionized water (DI) was prepared using a Millipore MilliQ system; the pH of the DI water should be no lower than ~6. Most reagent solutions, including the potassium phosphate buffer (50 mM, pH 7.4), glutathione (0.5 M), hydrochloric acid (HCl, 2 M), and sodium hydroxide (NaOH, 10 mM) were made fresh, no more than several days before use. In particular, the 0.5 M glutathione solution was prepared on the day of use. Pure stocks of HCl (concentrated, 35-37%), diethyl ether, methanol (MeOH, 100%) compressed dry N_2 (Ultrapure) were used. Bio-Rad AG 50W-X12 cation-exchange resin (H^+ form, 200-400 mesh; stored in 0.05 N HCl) was used for purification.

Before starting the experiment, the cation-exchange resin was cleaned to remove any contamination. The exchange column reservoir was loaded with 1 cm^3 of resin in between two plastic frits and placed on the Visi-prep manifold. 1 mL of DI water was added to the column and pulled into the resin using low vacuum pressure (<10 mbar) and allowed to sit for 15 min to hydrate by turning off the vacuum. 10 mL of DI water was then slowly pulled through the resin (i.e. dropwise, <10 mbar), followed by 10 mL of

pure methanol and a second 10 mL DI water rinse. The column was then loaded with 5 mL of 2 M HCl which was pulled into the resin and allowed to sit for 5 min. The resin was rinsed a final time with 10-15 mL of DI water to remove any remaining acidity. The pH of the eluent was checked with pH strips to ensure it was ~7. Resin cleaning should be performed within a week of use and properly stored by sealing the columns with Parafilm.

3.4 Protocol Specifics

The following protocol fully describes the required steps to synthesize and purify [^{35}S]-DMSP from commercial [^{35}S]-L-methionine. Schematic flow charts of the synthesis and purification (Figures 2 and 3) are provided for reference.

3.4.1 Enzyme-mediated Oxidation/Decarboxylation Reaction

The entire [^{35}S]-L-methionine stock was transferred from the manufacturer's vial to a 3 mL Reacti-vial (conical glass reaction vial). 0.5 mL of 50 mM potassium phosphate buffer (pH 7.4) was used to rinse the manufacturer's vial before adding it, 3.5 µmol of L-glutathione (7 µL of 0.5 M stock), and 1 unit of L-amino acid oxidase (~13-26 µL of stock) to the reaction vial. The vial was capped tightly, placed on a heat block at 37°C, and allowed to react for 24-30 h. The presence of glutathione in the reaction mixture is hypothesized to minimize the formation of a sulfoxide side product (A. Hanson, personal communication). After cooling to room temperature, the product [^{35}S]-MMPA was protonated by adding 40 µL of concentrated HCl. The solution was

transferred to a polypropylene (PP) centrifuge tube and combined with the product of 3x0.1 mL DI water rinses of the reaction vial, to ensure the transfer of all [^{35}S]-MMPA.

3.4.2 Diethyl ether Partitioning (a) and Base-Back Extraction (b)

To isolate the [^{35}S]-MMPA, several diethyl ether extraction steps were carried out. 3 mL of diethyl ether were added to the PP tube, the tube was vortexed for 1 min to thoroughly mix the phases, and then allowed to partition for 3 min. The top diethyl ether phase was extracted to a new PP tube using a glass Pasteur pipette, with care given to avoid the interface between the aqueous and diethyl ether phases. This procedure was repeated twice with 3 mL and once with 1 mL of diethyl ether, for a total of 4 extractions to ensure maximum recovery. All extractions were combined in a single PP tube.

Prior to proceeding with the methylation reaction, [^{35}S]-MMPA was deprotonated by adding 250 µL of 10 mM NaOH to the PP tube, the tube was vortexed for 3 min, and then the phases were allowed to separate for 5 min. The majority of the top diethyl ether phase was transferred into a new waste tube, taking care to avoid the interface between phases, in order to facilitate the next step. [^{35}S]-MMPA was extracted from the bottom aqueous phase using a new Pasteur pipette and transferred to a 3 mL Reacti-vial, again avoiding the interface as much as possible. The pH of the extracted [^{35}S]-MMPA product was tested by pipetting 0.5 µL onto a pH strip to corroborate that it was above 5 (often pH>7). If it was not sufficiently basic, the extraction with 250 µL of 10 mM NaOH was repeated before proceeding. 50 µL of 10 mM NaOH was then added to the PP tube to extract any remaining [^{35}S]-MMPA. The tube was vortexed again for 3 min, the

phases allowed to separate for 5 min, and the [^{35}S]-MMPA carefully removed and combined with the first extraction(s).

3.4.3 NaOH Phase Evaporation

The extracted NaOH phase containing the [^{35}S]-MMPA product was evaporated to dryness on a heat block at 35-40°C under a very gentle (< 60 mL min^{-1} in our system) stream of dry N_2 gas. Care was taken to prevent visible disturbance of the liquid's surface by the gas flow to minimize loss. Once the sample was fully evaporated, the [^{35}S]-MMPA product and any residue along the sides of the vial was reconstituted in 50 µL of DI water. At this point, the glass vial containing [^{35}S]-MMPA may be stored at -20ºC for short periods (i.e. less than a week) until further processing, but it is recommended to continue to the methylation reaction directly (section *3.4.4a*) to avoid losing activity to decay.

3.4.4 Methylation Reaction (a) and Acid Evaporation (b)

50 µL concentrated HCl and 25 µL of 20% (v/v) methanol in DI water were added to the glass vial containing the [^{35}S]-MMPA product. The vial was tightly capped and incubated for 25 min at 110°C in a heat block (Figure 2, section *3.4.4.a*). After the reaction time, the vial was removed from heat block and cooled to room temperature. The solution containing the [^{35}S]-DMSP product was evaporated to dryness at 35-40°C under a gentle stream of dry N_2, again taking care to avoid aerosolization (Figure 2,

section *3.4.4.b*). The product was reconstituted in 0.5 mL of DI water before continuing to purification. A 0.5 µL aliquot was taken to estimate the % [^{35}S] activity recovered after this step.

At this point, if the desired product is [^{35}S]-DMS, the unpurified [^{35}S]-DMSP can be acidified with 25 µL of 2 M HCl (~0.1 M final concentration) and preserved at -20^{o}C. This stock can then be used to generate [^{35}S]-DMS by adding base (e.g. 5 N NaOH) to an aliquot of the product in a sealed serum vial. [^{35}S]-DMS can then be removed using a gas-tight syringe and used for experimentation. Alternatively, the unpurified [^{35}S]-DMSP can be frozen for several days before proceeding with the purification steps, but it should not be acidified if the intent is to proceed with purification (Figure 3).

3.4.5 First Cation-Exchange Purification

A disposable exchange column containing 1 cm^3 of AG 50W cation-exchange resin (cleaned and rinsed, see section *3.3*) was placed in the solid-phase extraction manifold. Several muffled glass tubes (~11 mL capacity) were labelled numerically. The entire volume of product from the methylation reaction (section *3.4.4*) was pipetted into the column and drawn through the cation-exchange resin slowly, dropwise, under low vacuum pressure (i.e. <10 mbar) into collection Tube 1. The primary Reacti-vial was rinsed with 3x0.5 mL aliquots of DI water, adding each rinse to the column in turn. An additional 8 mL of DI water was drawn through the resin slowly to rinse any remaining contaminants. Tube 1 was replaced with Tube 2 and the cation-exchange resin was washed with an additional 4 mL of DI water.

The collection Tube 2 was replaced with Tube 3 prior to the elution of the [^{35}S]-DMSP main product, which was done with 8x1 mL aliquots of 2 M HCl. Each aliquot was pulled into the resin dropwise before adding the next one. The receiving tube was replaced one last time, and any remaining product was eluted with 4x1 mL of 2 M HCl into Tube 4. At all times, care was taken to ensure that the resin never ran dry without adding too much liquid to the headspace above it; the next aliquot was added once the previous was pulled into the resin. 5 µL aliquots were taken of each tube to quantify the radioactivity eluted in each step. Tube 1 and 2 may contain a significant amount of radioactivity due to the presence of byproducts and waste.

3.4.6 First HCl Evaporation

The product was concentrated from Tubes 3 and 4 by volatilizing the HCl, leaving behind the [^{35}S]-DMSP. When there was a significant portion of the [^{35}S]-DMSP product recovered in Tube 4 (e.g. >10%), the volume in Tube 4 was evaporated first, under similar conditions as the NaOH phase evaporation (at 35-40°C under a very gentle stream of N_2 gas; see section *3.4.3*). ~2 mL of the liquid from Tube 4 was added to a clean Reacti-vial using a muffled glass Pasteur pipette. The evaporation was monitored, topping off the liquid to keep the volume above 1 mL at all times. Tubes 3 and 4 were kept sealed with parafilm when not actively transferring product to minimize volatilization of [^{35}S]-DMSP. When the content of Tube 4 was fully transferred, the tube was rinsed with 0.5 mL of 2 M HCl and evaporated. The process was repeated with the remaining product solution contained in Tube 3. When the volume was transferred, Tube 3 was

also rinsed with 0.5 mL of 2 M HCl to recover any remaining [^{35}S]-DMSP product. At this point, the liquid in the Reacti-vial evaporated completely to dryness. The [^{35}S]-DMSP product was reconstituted in 90 µL of DI water and immediately taken through liquid chromatography purification (section *3.4.7*), though it can be preserved at <-20^{o}C overnight if needed.

3.4.7 Liquid Chromatography Purification

We used a Shimadzu LC-10AD liquid chromatograph (LC) outfitted with a methanol-cleaned Whartman Partisil 10 SCX column for [^{35}S]-DMSP purification. 50 mM KH_2PO_4 buffer (pH 7.4) was used as the mobile phase at a rate of 0.9 mL min^{-1}. The retention time of DMSP was established by performing a chromatography run with unlabeled DMSP, collecting 0.5 min eluate fractions into clean, muffled glass serum vials. The eluate from each time fraction was cleaved using 5 N NaOH, and of the presence of DMS was confirmed (e.g. via gas chromatography or a UV detector set to 190 nm). Special care was taken in rinsing the injection port and any material with DI to avoid contaminating the product of the radioactive synthesis with unlabeled DMSP and thus diminishing its specific activity.

The entire [^{35}S]-DMSP product (section *3.4.6*) was injected into the LC at one time, and fractions were collected every minute in clean, muffled glass serum vials, with a single vial used to collect the main [^{35}S]-DMSP fraction over the 2 min centered around the retention time. Before proceeding, the radioactivity in 0.5 – 1 µL aliquots from each fraction was counted to ensure that the [^{35}S]-DMSP was in the expected

serum vial. A quick, non-quantitative and non-destructive check of the presence of the ^{35}S label in the corresponding fraction can be performed by scanning the vials with a Geiger counter.

3.4.8 Second Cation-Exchange Purification

The [^{35}S]-DMSP containing fraction, product of the LC purification step (section *3.4.7*), was subjected to the same cation-exchange procedure as in section *3.4.5* to remove the KH_2PO_4 present in the mobile phase. A fresh column containing clean cation-exchange resin and muffled glass tubes were used. The radioactivity in 5 µL aliquots from Tube 3 and 4 were counted to quantify the amount of [^{35}S]-DMSP in each tube.

3.4.9 Second HCl Evaporation

As in section *3.4.6.*, the liquid from Tubes 4 and then 3 (including rinses of the primary collection tubes) were sequentially transferred to a clean, muffled Reacti-vial on a hot plate and evaporated at 35-40°C with a gentle flow of N_2.

3.4.10 Preparation of primary [^{35}S]-DMSP stock

When the final liquid addition was evaporated to dryness, the vial was cooled to room temperature, and the [^{35}S]-DMSP was reconstituted in 0.5 mL of 0.1 M HCl, the

volume of which was modified to target a specific [^{35}S]-DMSP activity (e.g. 2.5x10^{6} dpm mL^{-1}). If the final [^{35}S]-DMSP recovery was expected to be high or low, based on the % recovery from section *3.4.7*, the volume of 0.1 M HCl was adjusted higher or lower, respectively. A working stock was prepared with 2 µL of the primary [^{35}S]-DMSP stock in 998 µL of DI water, and 2 µL of this dilution was subsampled to quantify the amount of radioactivity and calculate the final yield of the synthesis. The primary stock of [^{35}S]-DMSP in HCl was stored at -20°C to reduce potential transformations (e.g. evaporation of liquid phase or degradation of DMSP).

As previously stated, if the desired product is [^{35}S]-DMS, the unpurified product from section *3.4.4* can be used to generate a working stock by diluting an aliquot of this unpurified product with DI water in a sealed serum vial and subsequently cleaving with base (e.g. 5N NaOH). Additionally, unpurified rinses from other steps, such as a rinse of the syringe used to inject [^{35}S]-DMSP into the LC column (section *3.4.7*), can be combined to make an unpurified stock of [^{35}S]-DMSP and subsequently used to generate [^{35}S]-DMS. [^{35}S]-DMS can also be generated from the purified [^{35}S]-DMSP product.

4. Results and Discussion

4.1 [^{35}S]-DMSP synthesis yield

Typical successful syntheses took approximately 2 weeks and used an initial ~5 mCi of [^{35}S]-L-methionine (specific activity 1100 Ci mmol^{-1}) to produce an average final yield of 5.34% (Table 2; n=16; range: 1.26% to 14.84%, excluding failures) of initial activity recovered as [^{35}S]-DMSP. For syntheses carried out in 2010, 2017, and 2021,

we can compare partial yields at each major step denoted in text (i.e. blue stars in Figures 2 and 3) and final yields (Table 2).The syntheses from these years were selected to show a wider range of final yields. The yield from the 2010 synthesis (13.8%) was on the high end of the range, while the other two (8.9 and 3.1%) are much closer to the average (5.34%). The yields from more recent years (i.e. since 2019) have been lower than earlier years likely due to the substitutions in materials (e.g. the resin used; see note on Table 2) and the passing of the original developer of the protocol. Notably, these more recent yields were still within the expected range of previous syntheses (data not shown).

Yields were calculated based on the subsamples indicated in the text and total volume of the main product fraction from that step. The volume in the tubes from the post-resin exchange were visually approximated, and the inherent human error involved likely led to an overestimation of the reactivity recovered and the % yield. As a result, it appeared that the yield increased from post-LC purification (section *3.4.7*) to post-resin exchange 2 (section 3.4.8), and artifact of the approximation methods. The volumes of the main product fractions from the other steps (sections *3.4.4, 3.4.7,* and *3.4.10*) were more precise, likely leading to less error in the calculations.

The protocol presented here, in general, has not been fully optimized for maximum purified [^{35}S]-DMSP yield. The decision not to further improve the methodology was twofold: (1) the lack of access to specialized instrumentation that could have reduced product loss (e.g., SpeedVac, see below), and (2) for the scope of our scientific inquiries, even a suboptimal synthesis yield still produced a sufficient quantity of [^{35}S]-DMSP to conduct DMSP tracer studies. Illustratively, [^{35}S]-DMSP turnover rate

constants are routinely obtained from incubations of 20 mL seawater with [^{35}S]-DMSP additions to target a concentration of 0.23 nCi mL^{-1} (Kiene and Linn, 2000b). The [^{35}S]-DMSP obtained from an average synthesis starting with 5 mCi [^{35}S]-L-methionine (5.34%; 270 µCi) would facilitate quantifying [^{35}S]-DMSP rate constants in nearly 60000 samples immediately following the synthesis. While a higher yield on average would be ideal for distributing [^{35}S]-DMSP among many groups, the availability of activity from a single synthesis for use in one laboratory would not, in most cases, be a limiting factor to facilitate study of DMSP cycling even 9 months after a synthesis (where ~10% of the original activity would still be available, facilitating ~6000 incubations, as described above).

[^{35}S]-DMSP chemically synthesized from commercially available [^{35}S]-L-methionine, as presented here, has been used repeatedly since its development in 2002, but its synthesis has never been described in detail to encourage broader implementation by the community. The multitude of investigations into the sulfur cycle that have used [^{35}S]-DMSP or [^{35}S]-DMS synthesized by the approach described here include, but are not limited to, quantification of microbial consumption rates of dissolved DMS and DMSP, characterization of the metabolic products of dissolved DMS and DMSP, quantification of DMS photolysis rates, and identification of organisms responsible for dissolved DMS and DMSP consumption. A non-exhaustive list of references related to the development and application of these analytical approaches is summarized in Table 1. Investigations into the DMSP/DMS cycle were performed in both *in situ* samples and a variety of plankton cultures with the [^{35}S] additions at tracer concentrations tailored to the specific application. The methods vary, though typically involve incubating spiked samples for a

given amount of time before parsing apart the [^{35}S]-labeled pool (e.g. into protein and DMS fractions), often via chemical transformations or isolations. Kiene and Linn (2000b) provide a good starting overview of fractions possibly quantified using this tracer.

4.2 [^{35}S]-DMSP Purification and Characterization

LC separation was the major purification step in the synthesis protocol, relying on a cation exchange column to do so. It separated a significant amount of radioactivity (Figure 4; up to 20-25% of the injected radioactivity, data not shown) from byproducts and reaction waste and confirmed that the main radioactive product was [^{35}S]-DMSP. It was also critically important to establish the retention time of DMSP in the LC system shortly before injecting the radioactive product. This helped confirm the presence of DMSP and allowed it to be collected in a single fraction. The system employed in this protocol was kept in open air, and fluctuations in room temperature influenced the retention time of DMSP. To help verify the presence of the target product [^{35}S]-DMSP, the retention time of non-radioactive DMSP was determined the same day of product purification under the same conditions. While the retention time of DMSP in LC systems with insulated columns in controlled environments would be more stable, the retention time should still be established using non-radioactive DMSP before the [^{35}S]-DMSP purification if one is used. There was a small absolute amount of [^{35}S]-DMSP recovered from the LC purification (216-1400 µCi, equivalent to 0.20-1.2 nmol; Table 2), which may not be perceived by the LC's UV detector.

The retention time of non-radioactive DMSP in our system was ~7.5 min (solid line, Figure 4). The sample loop in this LC system holds a volume of 100 µL; we dissolved our [^{35}S]-DMSP product (section *3.4.6*) in 90 µL of deionized to ensure one single injection with no loss. A single fraction was collected over 2 minutes centered

around the expected retention time of 7.5 minutes (6.5-8.5 minutes, Figure 4). This accounted for small shifts in the temperature of the column and the extended window of DMSP elution. There was a significant amount of byproduct (5.6-10.5%, data not shown) that eluted at a retention time of about 3 min. The characterization of this byproduct, and other possible byproducts from other steps (e.g. after the methylation reaction; step 3.4.4a), has not been performed. While analysis of these byproducts could aid in improving the yields of several steps, it is not necessary to verify the success of the protocol, and funding was not allocated to such formal analysis.

Evidence that the purified product was [^{35}S]-DMSP was further provided through DMS trap tests and uptake kinetics. Trap tests were performed, in which aliquots of a working stock of [^{35}S]-DMSP were cleaved using 5N NaOH, and the [^{35}S]-DMS produced was trapped onto a hydrogen peroxide-soaked filter with 95% efficiency (data not shown). Test uptake kinetics following the protocol from Kiene and Linn (2000a) in side-by-side incubations with and without glycine betaine (GBT, a known inhibitor of DMSP uptake; Kiene et al., 1998) also confirm the main product as [^{35}S]-DMSP.

4.3 Recommendations

Throughout the syntheses, care was taken to minimize loss and maximize recovered radioactivity at all possible steps. For example, after transferring the primary stock of [^{35}S]-L-methionine from the supplier's conical vial into the primary reaction vial, the supplier's vial was rinsed with the phosphate buffer to ensure that minimal activity

was lost. Similar measures were taken in all other steps that involve the transfer of the desired intermediary or product. The solvent evaporation steps (sections *3.4.6* and *3.4.9*) were likely sources of significant product loss. To combat this, evaporation steps were kept at slightly elevated temperatures (35-40°C) and low gas flow (< 60 mL min^{-1} for our system) with outlet tube height adjusted sufficiently off the surface of the liquid. Combined, these ensured that the surface of the liquid was not disturbed to prevent [^{35}S]-DMSP aerosolization and discourage product loss. They also reduced evaporation times and increased yields relative to open air evaporations at ambient conditions, though the difference has not been quantified. Any pure, dry, inert gas (e.g. argon or helium) could be used, though there likely would not be any benefit for the associated increased cost. While higher temperatures promoted faster evaporation rates, DMSP is heat-labile and will completely convert to DMS within hours at 70 °C (Hysert et al., 1979); tests have shown that approximately 22% of the [^{35}S]-DMSP product is lost at 50°C and greater than 95% at 90°C (data not shown). Others have managed to achieve higher final [^{35}S]-DMSP yields (>10%, S. Archer, personal communication) using solvent evaporation systems (e.g., SpeedVac SVC100 concentrator), but to our knowledge, no formal study on the effect such substitutions have on [^{35}S]-DMSP yield has been done.

The protocol has failed on several known occasions. It was hypothesized that the root cause of one was due to old or expired reagents, leaking lids, elevated reaction temperature ranges, or some combination therein. Based on protocols reported here, we recommend always using recently acquired primary reactants, making reagents shortly before use, and monitoring conditions closely to minimize any potential issues. Care should also be taken to avoid contamination of the extracted aqueous layer with

the ether phase during the transfer between tubes (section *3.4.2a*), as other syntheses have failed at this step.

The cation-exchange purification steps have been most successful when the vacuum pressure is kept low (i.e. <10 mbar) and the liquid pulled through dropwise. We observed that the more slowly the liquid was drawn through the cation-exchange resin, the greater the amount of [^{35}S]-DMSP was able to exchange onto the resin before elution with 2M HCl. The HCl solvent must then be completely evaporated before the LC step to concentrate the product to allow for a single injection and to adjust the pH of the sample injected into the LC column. This is the most time-consuming step (often 3-5 days) due to the slow evaporation rates of the 2M HCl, but it must not be rushed. The evaporation must be prevented from going to complete dryness until the end of the process to avoid blowing out the product. If needed, the procedure may be paused overnight by removing the vial from heat and capping it tightly. The decision to concentrate the product from Tube 4 should be based on the potential product recovery and the time associated. If the potential recovery is not greater than the loss due to radioactive decay, it is likely not worth volatilizing the HCl in Tube 4.

There are also several points in the protocol where the synthesis can be paused and the intermediary or unpurified products frozen before continuing (green and yellow octagons, Figures 2 and 3). However, experience has shown that the LC purification should immediately be followed by the second cation-exchange because DMSP is not stable in the elution buffer (50 mM KH_2PO_4). For experimentation in determining biogeochemical rates in biologic samples, we have traditionally targeted a concentration

of the purified [^{35}S]-DMSP stock of 2.5x10^{6} dpm μL^{-1} (~1.13 μCi μL^{-1}). The primary stock is concentrated to limit the amount of HCl added to live samples to ensure that the biological activity is not impaired by the presence of acid, specifically 1-2 half-lives (87-174 d) after the synthesis, when [^{35}S]-DMSP has decayed significantly, and larger volumes need to be added to reach similar activity levels in samples.

5 Conclusions

While not fully optimized for [^{35}S]-DMSP product formation, the procedure detailed here consistently yields significant quantities suitable for marine research purposes and has already been used successfully in a variety of research studies on DMS and DMSP biogeochemistry (Tables 1 and 2). Additionally, diversifying the isotopic labels beyond [^{35}S] (e.g. synthesis of L-[Methyl-^{3}H]-labeled DMSP) holds the potential to broaden its applicability further. The increased availability of radiolabeled DMSP to diverse research groups will facilitate further study, and presumably insight, into the kinetics and cycling of this globally important compound.

CRediT author contribution statement

A.M. Smith: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing- Original Draft, Writing- Review & Editing, Visualization, Project administration **D.A. del Valle:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing- Original Draft, Writing- Review & Editing, Project administration **A.R. Rellinger:** Methodology, Validation, Formal Analysis,

Investigation, Writing- Review & Editing, Project administration **J.W. Krause:** Resources, Writing- Review & Editing, Supervision, Project Administration, Funding acquisition **R.P. Kiene:** Conceptualization, Methodology, Writing- Original Draft, Validation, Investigation, Resources, Supervision, Project administration, Funding acquisition

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Application	Sample Type	DMSP concentration (nM)	DMS Concentration (nM)	[^{35}S] Tracer additions (pM)	Example Citations
Fate of S from DMS and DMSP	*In situ* DMS	1-7	-	2-4	del Valle et al., 2007
	In situ DMSP	1-5	1-5	<100	Royer et al., 2010
	Modified *in situ* DMSP	-	-	5.2	Motard-Côté and Kiene, 2015
Dissolved DMSP turnover rate	*In situ*	0-5	-	0.0011	Luce et al., 2011
	In situ	0.9-3.8	0-1.6	1.6-4.2	Li et al., 2016
	Diatom cultures	-	-	1.5-2.5	Lavoie et al., 2018
DMS turnover rates	*In situ*	-	0.25-27	<50	Kiene et al., 2007
	In situ	-	0.3-67.7	<1	del Valle et al., 2009
	In situ	0.8-2.6	0.8-4.7	1000*	Motard-Côté et al., 2016
Cell- and species-specific uptake rates of DMSP	Diatom Cultures	-	-	<100	Vila-Costa et al., 2006
	SAR11 Cultures	-	-	1.8	Tripp et al., 2008
Other	Diatom and dinoflagellate cultures	-	-	7000*	Saló et al., 2009
	Phaeocystis cultures	-	-	-	del Valle et al., 2011
	In situ	0-1.5	0.5-4	<50	Levine et al., 2016

Table 1: Selective list of published research applications of [^{35}S]-DMS and -DMSP obtained using the protocol detailed here. *In situ* samples consisted of natural communities except where noted; the tracer has also been used in plankton cultures. *In situ d*issolved DMSP and DMS concentrations are shown when available. Asterisks (*) denote tracer additions in dpm mL^{-1}.

Step		2010* Radioactivity Recovered (µCi)	2010* Yield (%)	2017* Radioactivity Recovered (µCi)	2017* Yield (%)	2021 Radioactivity Recovered (µCi)	2021 Yield (%)
Synthesis Start		5361	100	~5000	100	~5000	100
Post-Methylation (*3.4.4*)		ND	ND	1704.6	34.1	776.8	15.5
Post-Resin Exchange 1 (*3.4.5*)	Tube 3	1537	28.7	1113.0	22.3	597.0	11.9
	Tube 4	49	0.9	92.9	1.9	30.4	0.6
	Total	1586	29.6	1205.9	24.1	627.4	12.5
Post-LC Purification (*3.4.7*)		1421	26.5	510.5	10.2	216.4	4.3
Post-Resin Exchange 2 (*3.4.8*)	Tube 3	929.7	17.3	530.8	10.6	226.0	4.5
	Tube 4	2.6	0.0	0.14	0.0	0.1	0.0
	Total	932.3	17.4	530.9	10.6	226.1	4.5
Final Product (*3.4.10*)		**742**	**13.8**	**446.7**	**8.9**	**155.0**	**3.1**

Table 2: [^{35}S]-DMSP yields and % recovered relative to starting activity after the methylation reaction and specific purification steps for 3 syntheses from 2010, 2017, and 2021 using ~5 mCi of [^{35}S]-L-methionine.
ND: no data available.
* DOWEX 50W strong cation-exchange resin (H+ form, DOWEX 50W, 12% cross link, mesh size 200-400) was used, which is no longer available.

Figure 1: Reaction mechanism of the two required steps for the synthesis of DMSP. The first reaction (A) produces 3-methylmercaptopropionate from L-methionine, which is methylated in the second reaction (B) to produce 3-dimethylsulfoniopropionate.

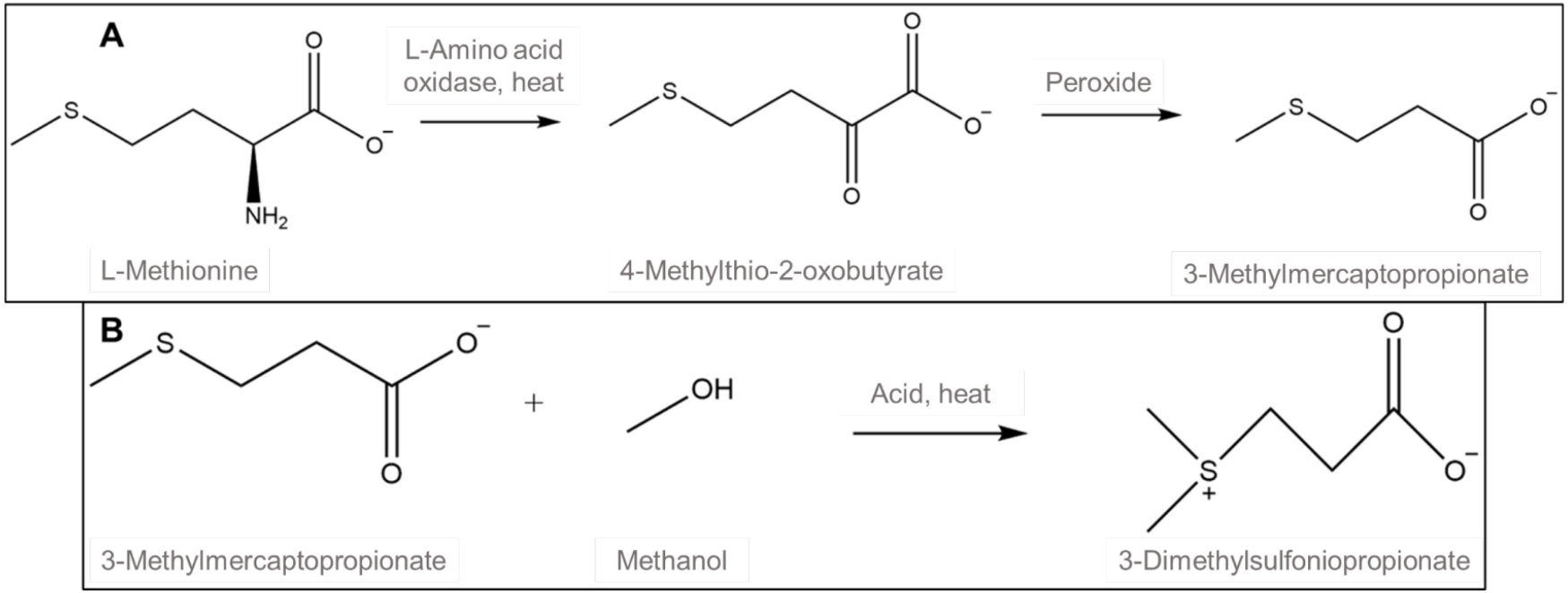

Figure 2: Flow chart of the steps required for the chemical synthesis of ^{35}S-DMSP and DMS from [^{35}S]-L-methionine. The labeling of the steps corresponds to those described in the methods. Octagons indicate places where the synthesis can or cannot be paused: yellow indicates where it is recommended to continue the procedure (after step 3.4.3) and green indicates where it has not been shown to affect yield (after step 3.4.4b). Blue star indicates where an aliquot was taken to quantify radioactivity and % radioactivity recovered at that step (see Table 2).

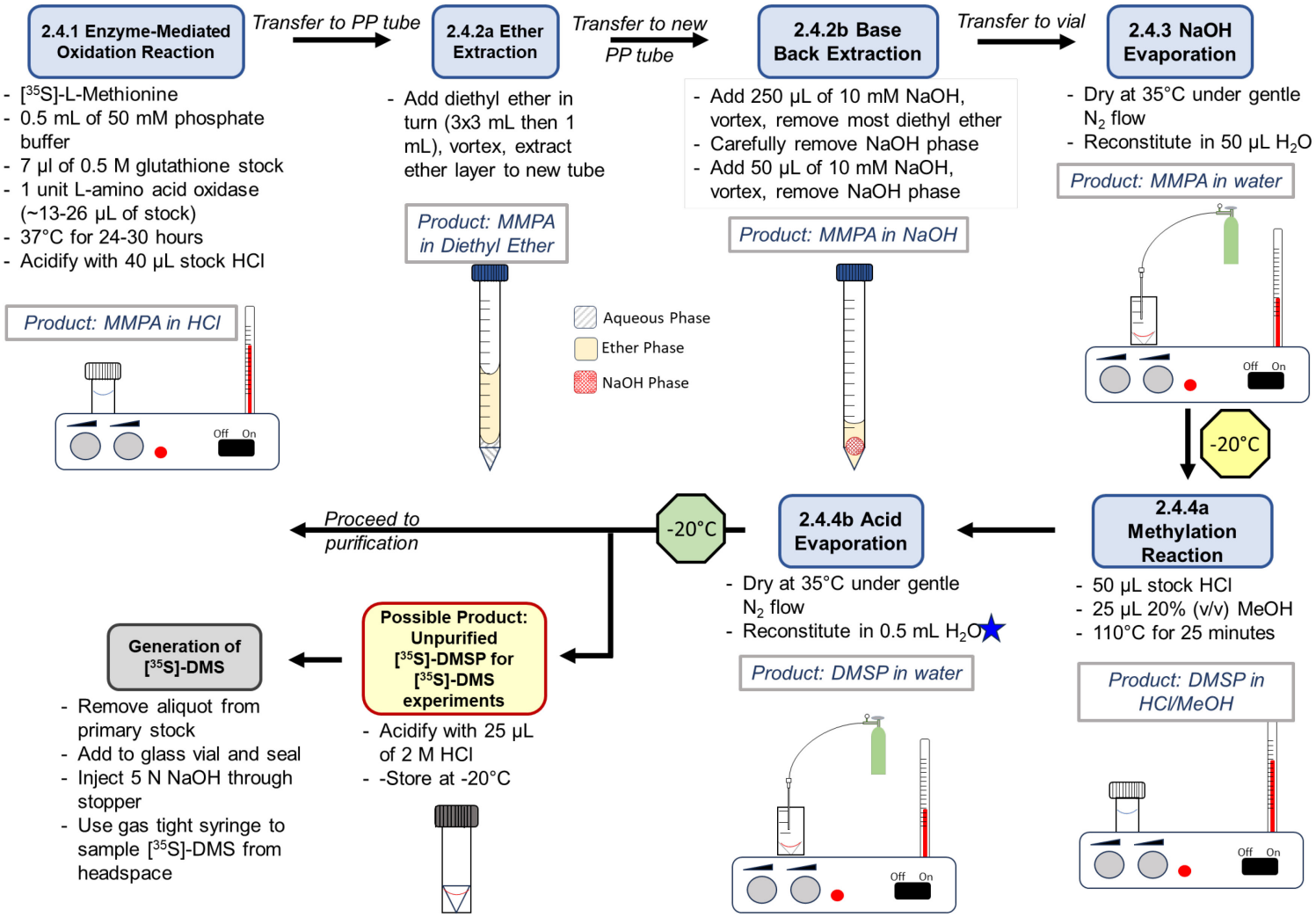

Figure 3: Flow chart of the steps required for the purification of [^{35}S]-DMSP . The labeling of the steps corresponds to those described in the methods. Octagons indicate places where the synthesis can or cannot be paused: green indicates where it has not been shown to affect yield (after step 3.4.6) and red indicates where the protocol should not be paused (after step 3.4.7). Blue stars indicate where aliquots were taken to quantify radioactivity and % radioactivity recovered at each step (see Table 2).

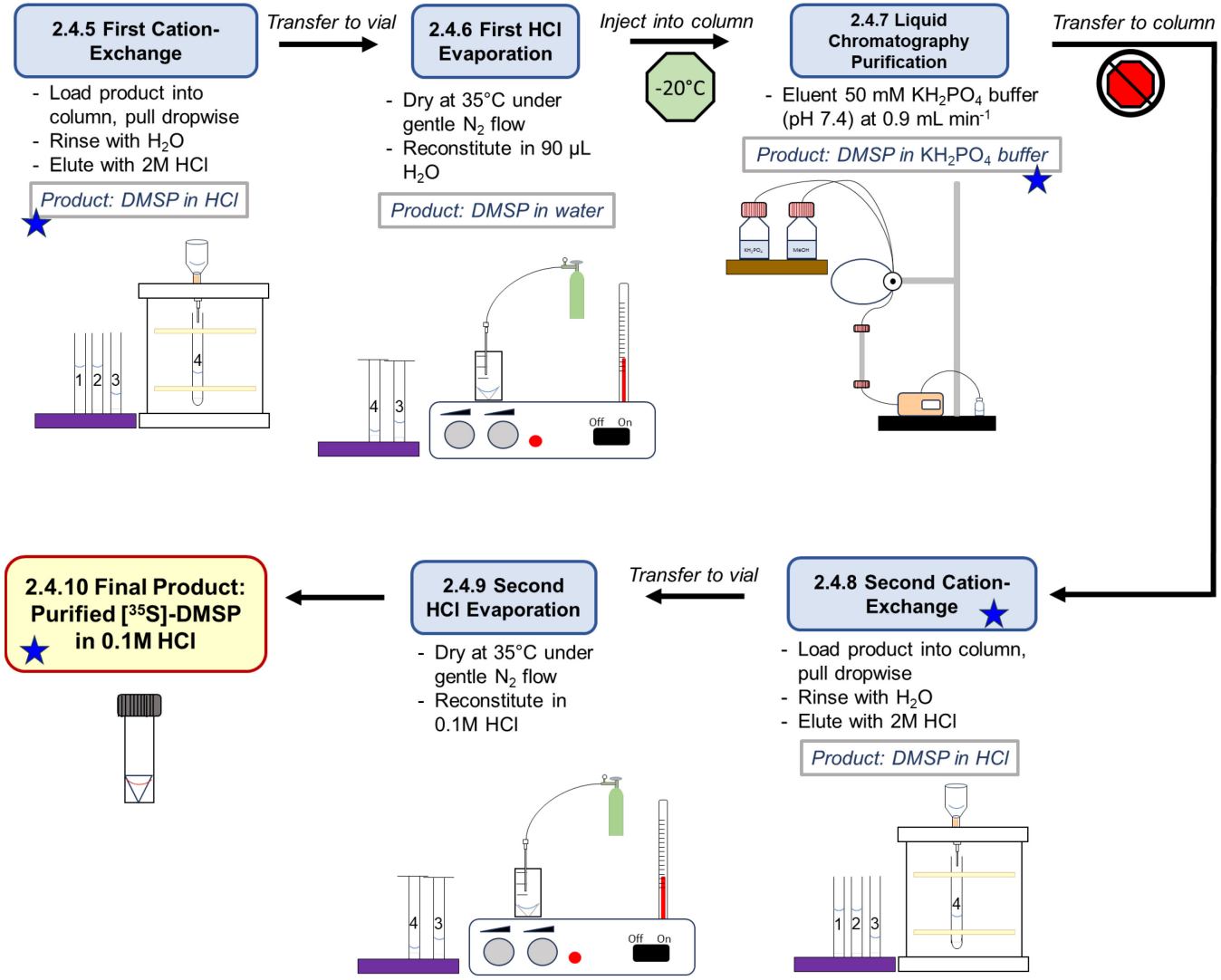

Figure 4. LC chromatogram of unlabeled DMSP (circles, solid line) and [35S]-DMSP (squares, dashed line) obtained during the 2017 synthesis. The retention time of unlabeled DMSP was determined by cleaving to DMS using base and measuring by gas chromatography in each collected time fraction. The square root of the peak area of the gas chromatogram is directly linearly correlated to the amount (i.e. mol) of DMS in the sample. After the injection of the [35S]-DMSP product (section 2.4.7), total radioactivity in each time fraction was quantified in 10 µL subsamples.