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Direct quantification of dimethylsulfoniopropionate (DMSP) with hydrophilic interaction liquid chromatography/mass spectrometry

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ABSTRACT

A simple, derivatization free method for the direct determination of dimethylsulfoniopropionate (DMSP) using hydrophilic interaction liquid chromatography (HILIC)/mass spectrometry is introduced. DMSP is a zwitterionic osmolyte which is produced from marine plankton, macro algae and higher plants. Due to its central role in climate relevant geochemical processes as well as in plant physiology and chemical ecology there is a great interest in methods for its quantification. Since DMSP is labile and difficult to extract currently most protocols for quantification are based on indirect methods. Here we show that ultra performance liquid chromatography/mass spectrometry using a HILIC stationary phase is suitable for the direct quantification of DMSP from aqueous samples and microalgal extracts. The protocol requires minimal sample preparation and phytoplankton samples can be investigated after filtration of small volumes. The limit of detection is 20 nM and the calibration curve is linear in the range of 60 nM to 50 μM. The use of [$^2H_6$]-DMSP as internal standard allows prolonged sample storage since it is transformed with the same kinetics as natural DMSP. This makes the method suitable for both laboratory and field studies.

1. Introduction

Dimethylsulfoniopropionate (DMSP) is a sulfur-containing metabolite produced by many marine micro and macro algae. The zwitterionic compound can serve as an antioxidant [1], osmolyte and cryoprotectant [2]. DMSP can be transformed by the enzyme DMSP-lyase to acrylic acid and the volatile dimethylsulfide (DMS) [3]. In addition, a second acrylic acid independent DMS release mechanism from DMSP by bacteria is reported [4]. Besides its physiological functions DMS serves as an infochemical and can influence interactions in the plankton over several trophic levels [5]. Along with acrylic acid DMS plays a role in algal chemical defense against grazers [6–8]. DMSP has also been detected in several higher plants where its role in plant physiology and as source of off flavors is intensively studied [9,10]. DMSP is considered to be the major precursor of DMS that is emitted in high quantities of 13–37 Tg sulfur per year into the atmosphere. This corresponds to 90% of the biogenic sulfur emissions from the oceans and almost 50% of the biogenic sulfur emissions worldwide [11]. In the atmosphere DMS is oxidized to form sulfate containing aerosols that can serve as cloud condensation nuclei and thereby influence climate processes [12,13].

Due to the central importance of DMSP in geochemistry, ecology, and algal or plant physiology several methods have been developed for its quantification in different matrices. In most studies DMSP is determined indirectly using base mediated cleavage of DMSP to DMS and acrylate. DMS can then be analyzed via GC using headspace or SPME techniques [14]. But since other potential DMS precursors are found in several species of macro algae and phytoplankton [15–19], these additional DMS sources lead to an overestimation of DMSP when indirect analytic methods are utilized. In addition, quantification of the volatile can be cumbersome due to the requirement for an initial chemical reaction for DMS release and the handling involved in headspace techniques. Different approaches have thus been developed for the analysis of DMSP. Capillary electrophoresis of the *p*-bromophenylacylester of DMSP was used to analyze DMSP in macroalgal and plant samples [20]. Pyrenyldiazomethane was used for preparation of DMSP esters from micro- and macroalgae for UPLC/MS measurements [21]. Comparably few methods have been reported for the direct determination of DMSP. Only recently Li et al. [22] introduced a LC/MS method for direct determination of DMSP and other betains using a pentafluorophenylpropyl column with a ternary gradient. They optimized the protocol for analysis of coral tissue extracts. A HPLC/UV method for the detection of DMSP in samples from perchloric acid extracts of higher plants using ion chromatography was reported by Colmer et al. [23]. This method was developed for the investigation of sugar cane and used UV absorbance at 194 nm for

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detection. However this might result in problems in selectivity and sensitivity due to the lack of a chromophore of DMSP and due to the high amount of matrix components in comparably complex marine samples. Due to the zwitterionic structure and low molecular weight analysis of DMSP with common reversed phase (RP) LC systems is not possible as the analyte elutes with the void volume.

We reasoned that hydrophilic interaction liquid chromatography (HILIC) that facilitates the analysis of polar compounds might be suitable for retention of the underivatized zwitterionic DMSP and the detection of this metabolite in plankton samples. This led to the development of an easy and sensitive method for the direct determination of DMSP using HILIC with LC/MS, which could be readily applied for the analysis of phytoplankton samples.

2. Experimental

2.1. Reagents

Anhydrous acrylic acid, dimethylsulfide and [$^2H_6$]-dimethylsulfide were purchased from Sigma–Aldrich (Germany). Acetonitrile and water (UPLC/MS grade) were purchased from Biosolve (Netherlands).

2.2. Equipment

For separation an Acquity^{TM} ultraperformance LC (Waters, Milford, MA, USA) equipped with an Aquity UPLC^{TM} BEH HILIC column (1.7 μm, 2.1 mm × 50 mm) was used. The module was coupled to a Q-ToF Micro mass spectrometer (Waters Micromass, Manchester, England).

2.3. Synthesis

DMSP and [$^2H_6$]-DMSP were synthesized according to Chambers et al. [24]. Crude products were recrystallized from methanol/diethylether (1:2, v/v) with yields of 68% and 74%, respectively (NMR data [21]). ^{1}H NMR revealed that purities of 95% were obtained. LC/MS analysis revealed that no detectable unlabeled DMSP is present in [$^2H_6$]-DMSP samples (Fig. 1b).

2.4. Method development

For UPLC separation an eluent system of water + 2% acetonitrile (solvent A) and acetonitrile (solvent B) was used. Separation started with 10% A and a flow rate of 0.25 ml min^{-1} for 0.40 min. The gradient was set to 60% A at 0.41 min and held at this ratio till 1.70 min. To accelerate washing, the flow rate was increased to 0.60 ml min^{-1} within 0.20 min. This setting was kept for 0.75 min, and then flow rate and gradient were set back to 0.25 ml min^{-1} and 10% A. The column was equilibrated for 1.30 min, resulting in a total analysis time of 4 min. Injection of the sample solution (1–5 μl) was performed using a loop injector. The auto sampler temperature was held at 4 °C, the column temperature was set to 27 °C. Mass measurements were performed in the ESI-positive mode, recording the mass range from 105 to 200 m/z using a scan rate of 0.6 s and an inter-scan delay of 0.1 s. The following MS parameters were applied: capillary voltage 3000 V, sample cone 10.0 V, source temperature 120 °C, desolvation gas temperature 300 °C, collision energy 5.0 V, collision gas argon, ion energy 1.8 V. The resolution of the ToF-MS was 6000. For qualitative MS/MS analysis collision energy was set to 10 V with a scan rate of 0.5 s.

2.5. Cultivation

Unialgal cultures of the diatoms *Skeletonema costatum* (RCC75) and *Thalassiosira weissflogii* (RCC76) and the coccolithophore *Emiliania huxleyi* (CCMP1516) were propagated in autoclaved medium at 16 °C (RCC76 at 13 °C) with a 14:10 light:dark cycle. Artificial seawater for diatom cultures was prepared as described by Maier and Callenberg [25]. Medium for cultures of *E. huxleyi* was prepared by dissolving 33.33 g l^{-1} HW sea salt professional (aquaristic.net, Babenhausen, Germany) in double distilled water. 400 μl of separately autoclaved Seramis® for foliage plants (MARS GmbH, Mogendorf, Germany) were added to this seawater preparation. Light was provided by Osram biolux lamps with an intensity of 40 μmol photons m^{-2} s^{-1}.

2.6. Sample preparation

Standard solutions of DMSP and [$^2H_6$]-DMSP were prepared in water/acetonitrile (10:90, v/v). To adjust salinity 40 μl of filtered artificial seawater [25] were added to 1 ml of the water/acetonitrile solutions and the samples were centrifuged (5 min, 16,000 × g).

Microalgal cultures were counted in a Fuchs-Rosenthal haematocytometer and (50 ml of 3×10^6 cells ml^{-1} *E. huxleyi*, 50 ml of 8×10^6 cells ml^{-1} *S. costatum* and 150 ml of 6×10^5 cells ml^{-1} *T. weissflogii*) gravity filtered on Whatman GF/C. The filters with the cells were transferred into 1.5 ml of methanol containing 100 μl of a 200 μM [$^2H_6$]-DMSP solution as internal standard. After 6 days at −20 °C 100 μl of these extracts were diluted with 900 μl water/acetonitrile (10:90, v/v) and centrifuged (5 min, 16,000 × g). The supernatants were directly used for UPLC analysis.

2.7. Linearity, quantification and precision

Two concentration ranges were tested for linearity. Solutions of 0.5, 0.75, 1, 5, 10, 15, 20, 25, 30, 40 and 50 μM were used with a final concentration of 2 μM [$^2H_6$]-DMSP as internal standard. Solutions of 60, 80, 100, 200, 300 and 400 nM were used with a final concentration of 200 nM [$^2H_6$]-DMSP as internal standard. The area ratio of DMSP (135m/z) and [$^2H_6$]-DMSP (141m/z) was plotted relative to the DMSP concentration. A 0.05 amu mass window was adjusted around the respective molecular ion. The limit of detection (LOD) and quantification (LOQ) were defined as the amount DMSP required to reach a signal to noise ratio higher than 3 and 10, respectively. Intra-day and inter-day precision was determined by repeated injection (n = 6) of three standard solutions (100 nM, 1 and 10 μM) within 1 day or on 4 days within one week. Inter-day precision was also determined for algae samples (injection on 2 days within one week).

2.8. Effects of sample stability on quantification

The stability of standard solutions was verified by injecting the same sample after 0, 14, 24, 92 and 164 h after preparation. During the first 24 h samples were stored at 4 °C in the auto sampler of the LC/MS. Long term storage was conducted at −20 °C.

Stability of algae extracts in water/acetonitrile was determined by injecting the samples 0 and 72 h after dilution. Samples were stored at −20 °C between measurements.

To test the stability of algae extracts stored at −20 °C a methanol extract (2 ml) of *T. weissflogii* (RCC76) was spiked with 20, 50, 100 and 200 nmol DMSP and 20 nmol [$^2H_6$]-DMSP. This culture did not contain any detectable levels of DMSP, so no interference with a natural DMSP background could occur. Samples were stored for 7 months and analyzed as described above.

3. Results and discussion

3.1. Method development

The composition and flow rate of the mobile phase were optimized to give best results in peak shape using a synthetic DMSP

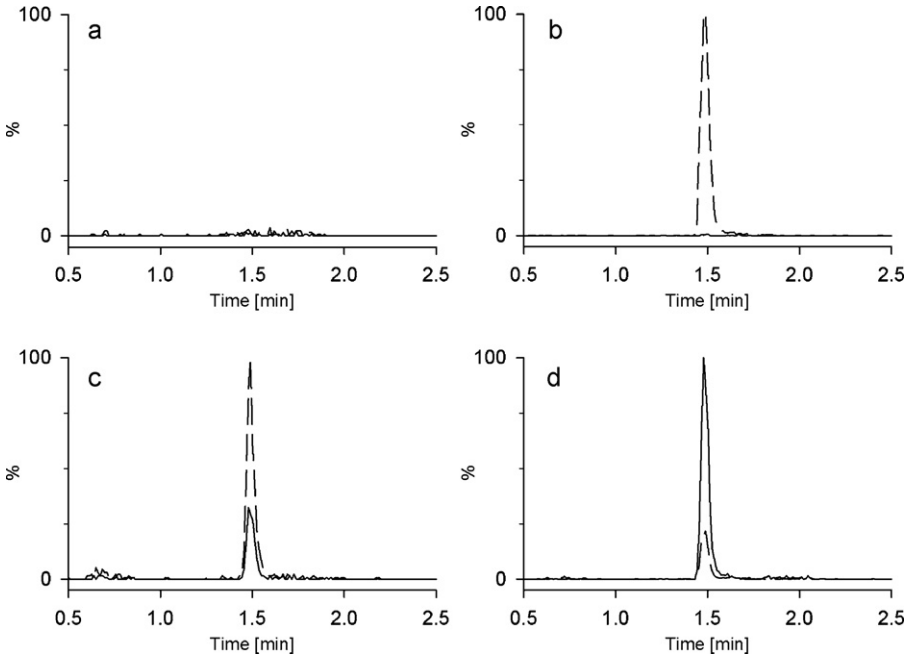

Fig. 1. LC/MS-chromatogram of (a) blank solution (water/acetonitrile and seawater), (b) solution of 4 μM [$^2H_6$]-DMSP (1 μl injection), (c) DMSP standard solution (60 nM DMSP, 200 nM [$^2H_6$]-DMSP, 5 μl injection) and (d) methanol extract of *S. costatum* with added standard (1.29×10^6 cells ml^{-1}, 42 ml filtration volume, 1:10 diluted, 1 μl injection). The total ion current was recorded, the solid line shows the ion trace for DMSP detection (135*m*/*z*), and the dashed line the ion trace for [$^2H_6$]-DMSP detection (141*m*/*z*). Intensity in (a) was normalized to the intensity of [$^2H_6$]-DMSP in (c).

standard in distilled water/acetonitrile. Surprisingly algal samples resulted in more intense peaks with better peak shapes compared to the standard solutions. In accordance, addition of seawater to the standard solution resulted in enhanced sensitivity and better peak shape. For a systematic evaluation of the effect of salts, the salinity of the sample solution was increased by adding different proportions of seawater to the standard solutions. Without seawater addition to the standard solution DMSP eluted at 1.56 min in a broad peak. Addition of 10, 20 or 40 μl seawater led to a gradual shift of the retention time to 1.47 min and sharper peaks. The same retention time was also obtained for algae samples from seawater (Fig. 1d). Higher salt concentrations were avoided to minimize washing time for removal of polar components. These remaining impurities on the column material influence the peak shape remarkably. When insufficient washing was conducted, the DMSP signal showed extensive tailing with a second maximum at 1.56 min after repeated injection of the same sample. This might be due to the silica gel based column material, which shows high affinity to polar components that reduce the separation potential for the zwitterionic DMSP. It became evident that extending the washing time with water/acetonitrile (60/40) was required to guarantee a constant quality during the measurement of extended sample series. But to shorten time required for washing the flow rate could be doubled (0.60 ml/min) compared to the flow rate during separation during the wash step of the run. Addition of formic acid to the eluent decreased both sensitivity and quality of the peak shape with and without added salts. Thus no further additions of modifiers to the eluents were made.

To reduce the risk of detecting other seawater or extract components than DMSP and the standard [$^2H_6$]-DMSP, the ion trace of the respective molecular mass with a narrow mass window of 0.05 Da was used for quantification. A blank sample containing water/acetonitrile and seawater did not show any signal for the respective mass traces (Fig. 1a). However, if required peak analysis without interference of potential contaminants can also be supported by MS/MS-techniques. DMSP and [$^2H_6$]-DMSP show characteristic fragments at 73*m*/*z* for acrylic acid and 63 and 69*m*/*z* for DMS and [$^2H_6$]-DMS, respectively (Fig. 2). Since no comparable fragments were observed in natural samples MS/MS might be an option for selective DMSP determination. In our case no interference with other components of the samples was observed and we thus did not revert to MS/MS experiments for quantification.

3.2. Linearity, detection limit and precision

The ratio of peak areas and DMSP concentration was linear over the range of 0.06–50 μM (Mandel adaptation test [26]). Using an injection volume of 5 μl the LOD and LOQ was 20 and 60 nM (Fig. 1c), respectively. Higher injection volumes did not improve the LOD. Repeated injection of a 60 nM standard solution revealed a measurement precision <10% and accuracy between 80 and 120%. We selected the two different concentrations of 2 μM and 200 nM of the internal standard [$^2H_6$]-DMSP to generate two calibration curves that span the entire concentration range. The calibration curve was: $y = 417.0 \times 10^{-3}x - 7.2 \times 10^{-3}$ (x corresponds to DMSP in μM) and $y = 4.4 \times 10^{-3}x + 7.4 \times 10^{-3}$ (x corresponds to DMSP in nM) with a correlation coefficient (r^2) >0.999 and >0.995 for the calibrations using 2 μM and 200 nM internal standard, respectively. Intra-day precision was 5.5% for 100 nM, 7.2% for 1 μM and 6.1% and 10 μM DMSP concentrations. Inter-day precision was 6.1% for 1 μM and 4.8% for 10 μM DMSP concentrations. For algae samples of *S. costatum* and *E. huxleyi* an inter-day precision of 7.8% and 7.5% were found.

3.3. Effects of sample stability on quantification

Methanolic extracts of *T. weissflogii* samples that were spiked with 20, 50, 100 and 200 nmol DMSP and 20 nmol [$^2H_6$]-DMSP, were stable even after prolonged storage (7 months) at −20 °C. Here, recovery rates of 95–105% for DMSP were found. The samples in water/acetonitrile showed reduced stability over a prolonged time of storage which was reflected by reduced peak areas. Already after 24 h samples with initially 100 nM DMSP concentration could not reliably be quantified. The degradation mechanism is unknown,

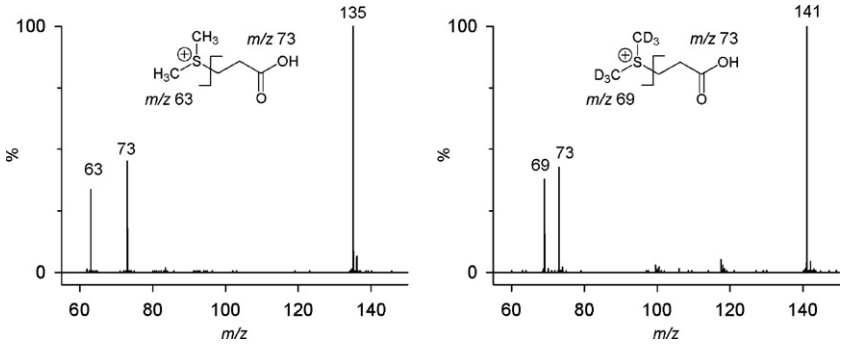

Fig. 2. MS/MS spectra of 20 μM DMSP (left) and 20 μM [$^2H_6$]-DMSP (right); average over 4 scans (background corrected).

however, even after 7 days >90% of the initial DMSP concentration for the 1 and 10 μM standards was found. The high recovery rate is the result of using deuterated DMSP as internal standard as a similar decrease of peak intensity was observed for both deuterated standard and analyte over the entire measurement time. Therefore, a kinetic isotope effect can be excluded for degradation of labeled DMSP. This is supported by the fact that the bond that is broken during DMSP cleavage is not immediately affected by the remote deuterium substitutions. Due to the limited stability samples should thus be prepared directly before each measurement series. We recommend to prepare methanolic extracts containing the internal standard that can be stored at −20 °C, e.g. during field experiments or long term studies as these extracts are stable over 7 months. These methanolic solutions should only be diluted with water/acetonitrile right before the measurement.

3.4. DMSP in microalgae

The method was applied for the determination of the particulate DMSP concentration in phytoplankton cultures. Particulate DMSP refers to the DMSP content of plankton samples which are usually obtained by filtration on GF/C as it is also performed in the method introduced here. Only few ml of phytoplankton cultures are sufficient for the DMSP analysis. For the diatom *S. costatum* 0.21 pg DMSP/cell, and for the coccolithophore *E. huxleyi* 0.60 pg DMSP/cell were found. Referred to cell volume the concentrations were 23.8 mM for *S. costatum* and 52.0 mM for *E. huxleyi*. The values are well within the range of those observed for microalgae, and differences between the results presented here and previously reported DMSP cell contents are probably due to different culture conditions employed (temperature, aeration) [27].

In the surface ocean particulate DMSP concentration is a relevant factor if plankton interaction and atmospheric chemistry is concerned. These concentrations vary widely from below 2 nM [28] to >500 nM during alga blooms in the open ocean [29]. Due to the low LOQ and the wide linear range the method introduced here can be applied for both scenarios after filtration of several milliliters to few liters of plankton samples. The LOD of our method is improved compared to methods using derivatization, which is most likely due to the lack of an additional chemical transformation step [21]. The headspace methods with LOD down to 0.03 nM are however more sensitive than the direct determination introduced here [14]. An RP-HPLC/MS method on a pentafluorophenylpropyl column that was optimized for the investigation of coral tissue possesses a three times lower LOD than the HILIC–HPLC/MS method introduced here [22]. In contrast to the Li et al. [22] method we only require a binary gradient and achieve shorter times for analyses. Our protocol relies on a completely different sampling strategy compared to [22] making organisms from the dilute matrix of the marine plankton accessible for direct DMSP quantification. It is not only applicable to laboratory studies but also to samples isolated during research cruises or other field work since the addition of the internal standard right after filtration compensates for DMSP degradation during storage and transport.

4. Conclusion

We developed a UPLC/MS method for the direct determination of DMSP. The HILIC/MS method allows a selective detection of the polar analyte. For sample preparation only a dilution of the extract with water/acetonitrile and centrifugation is required. In comparison to other available methods a big advantage lies in the fact that no additional steps like derivatization, purification or chemical transformation are required. Headspace methods using base mediated release of DMS suffer in addition from the risk of an overestimation due to alternative DMS sources than DMSP. This risk is prevented by the direct determination of DMSP without any additional chemical transformation. Due to the low LOD the method can be directly applied for both alga and phytoplankton samples. Methanol cell extracts can be stored for prolonged time which makes our protocol suitable for field studies and long term surveys.

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