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Direct quantification of dimethylsulfoniopropionate (DMSP) in marine micro- and macroalgae using HPLC or UPLC/MS

Theresa Wiesemeier, Georg Pohnert *

Laboratory of Chemical Ecology/Institute of Chemical Sciences and Engineering/École Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

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Abstract

A simple method for the direct quantification of dimethylsulfinopropionate (DMSP) using HPLC or UPLC coupled to UV and/or MS detection is introduced. The protocol is applied for the determination of DMSP from marine micro- and macroalgae. The method is based on the derivatisation of DMSP using 1-pyrenyldiazomethane followed by reversed phase HPLC or UPLC separation. The detection limit is 590 nM, corresponding to 1 ng DMSP per injection. Using a combination of UV and MS detection the calibration curves were linear in the range of 2.93 μM to 11.7 mM concentrations. We show that direct determination of DMSP is possible from macroalgal tissue and microalgal cultures if DMSP-lyase activity is suppressed during work-up.

Keywords: HPLC/MS; UPLC/MS; Marine plankton; Dimethylsulfide (DMS); Dimethylsulfinopropionate (DMSP)

1. Introduction

Dimethylsulfoniopropionate DMSP is an osmolyte and antioxidant produced by phytoplankton, macroalgae and submerged aquatic plants [1,2]. There is considerable interest in this tertiary sulfonium compound since it is present at significant concentrations (5 to >200 nM) in the surface ocean [1,3,4]. DMSP is the major precursor of dimethylsulfide (DMS), which is released by the enzyme DMSP-lyase from micro and macroalgae with acrylic acid as second cleavage product (Fig. 1) [5]. DMS release from DMSP may also be initiated by bacterial degradation or via a base mediated reaction [1,6]. DMS is one of the most important biogenic sources of atmospheric sulphur and contributes 17–34 Tg S year^{-1} which represents more than 90% of the biogenic sulphur emissions from oceans and roughly half of the total biogenic sulphur emission [5]. Oxidation products of DMS form sulphate aerosols initiating cloud formation and thereby influencing climate regulation [7].

Besides its substantial biogeochemical significance, DMSP plays also an important role in the mediation of ecological interactions. It is involved directly or after cleavage to DMS and acrylate in the chemical defence of micro- and macroalgae [1,8–12]. Even if it was suggested that DMSP is essentially non-toxic [13,14] further investigations showed that DMSP itself or its break down products could function as a feeding inhibitors for herbivores [14,15].

Despite this considerable interest in DMSP, there are few direct methods available for its determination from aqueous or tissue samples. The most commonly used methods for detection of DMSP rely on the indirect determination of gaseous DMS, which is released upon treatment with cold, concentrated alkali. Using different techniques DMS thus liberated from DMSP is commonly quantitatively analysed by gas chromatography [3,4,16–21]. A substantial problem of these indirect methods is that they might lead to an overestimation of DMSP since DMS may also be produced from a variety of different biogenic precursors [22–24]. To overcome this problem HPLC/UV based methods were developed for the direct quantification of DMSP [23,25,26]. But due to the low UV-absorption of the analyte and the chromatographic properties of this zwitter ion significant interference with co-eluting signals renders these approaches unreliable if no preliminary purification is performed. Recently, an efficient capillary electrophoretic based quantification protocol for DMSP was reported, but since this technique is not

* Corresponding author. Tel.: +41 21 6939357; fax: +41 21 6939305.
E-mail address: georg.pohnert@epfl.ch (G. Pohnert).

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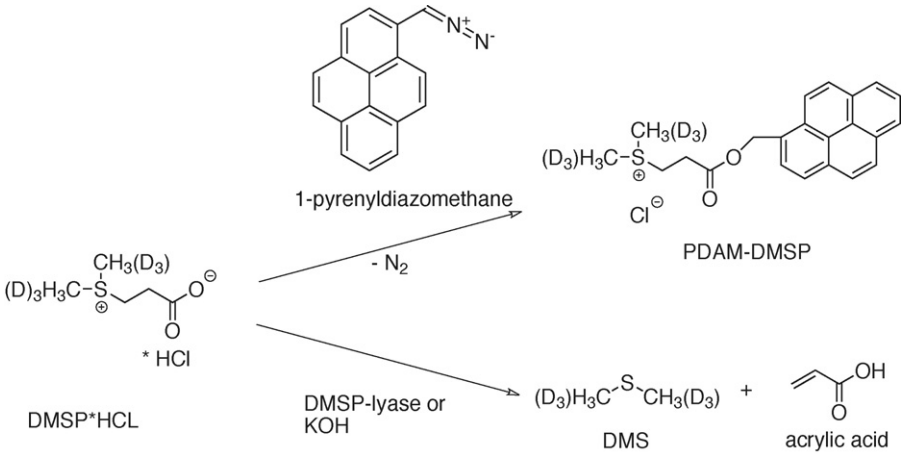

Fig. 1. Derivatisation of DMSP with 1-pyrenyldiazomethane and enzymatic DMSP-cleavage.

widely distributed its application will most probably be limited to specialized labs [27]. Here we introduce a direct UPLC (ultra performance liquid chromatography)/MS method for identification and quantification of DMSP after derivatisation. We demonstrate that the method can also be easily adapted for DMSP – quantification using basic equipment, such as a HPLC with UV detector. The method is based on an initial derivatisation of DMSP with 1-pyrenyldiazomethane (PDAM), which reacts with carboxylic acids forming fluorescent and stable esters [28]. It was applied for the investigation of samples from marine micro and macroalgae (Fig. 1).

2. Experimental

2.1. Reagents

Dimethylsulfide, [$^2H_6$]-dimethylsulfide, anhydrous acrylic acid and sodium chloride were purchased from Aldrich/Fluka (Buchs, Switzerland). Hydrochloric acid and methanol were purchased from Merck (Dietikon, Switzerland). Sulphuric acid and dichloromethane were obtained from Fisher Scientific (Switzerland). For derivatisation 1-pyrenyldiazomethane (PDAM) from Sigma/Aldrich (Buchs, Switzerland) with a concentration of 1 mg/ml $CHCl_3$/MeOH (3:1) was used unless otherwise indicated.

Artificial seawater for rearing of macroalgae was prepared by dissolving 33 g/l Instant Ocean (Aquarium Systems, Sarrebourg, France) in distilled water. Seawater medium for *Emiliania huxleyi* cultures was prepared as described by Maier and Calenberg [29].

2.2. Equipment

Separation was performed using an Acquity^{TM} Ultraperformance LC (Waters, Milford, MA, USA) with a 2996 PDA detector equipped with an Acquity UPLC^{TM} BEH C_{18} column (1.7 μm, 2.1 mm × 50 mm) or a Grom-Sil 120 ODS-3 CP column (3 μm, 2 mm × 125 mm). This module was coupled to a Q-tof Micro mass spectrometer (Waters Micromass, Manchester, England). For ^{1}H NMR and ^{13}C NMR measurements a Bruker Avance 400 NMR spectrometer was used.

2.3. Derivatisation of DMSP

For derivatisation of the standard a solution of 1 mg DMSP *HCl in 1 ml MeOH (pH 4.5) was treated with 2 ml of an 8 mg/ml solution of PDAM in $CHCl_3$/MeOH (3:1). The resulting solution with a pH of 5.3 was incubated for 1 h at room temperature. The pH of the medium should not reach alkaline pH to avoid DMSP-cleavage under basic conditions [6]. After derivatisation the solvents were removed under a stream of nitrogen and the residue was taken up with 1 ml of MeOH. Samples were centrifuged, diluted to reach concentrations required for the determination of linearity, detection limits and quality control (see below) and used directly for HPLC/MS or UPLC/MS investigations. Control measurements were run as outlined above without addition of DMSP *HCl.

2.4. Method development

For UPLC separation the sample solution (1–10 μl) was injected into a flowing stream of water + 0.1% acetic acid (solvent A) and methanol/THF (80:20) + 0.1% acetic acid (solvent B) at a ratio of 60:40 with the flow rate 0.6 ml/min using a loop injector. Starting with this solvent ratio a gradient was ranged to 60% solvent B (1 min) and then to 100% solvent B (2.50 min). After 3 min the column was re-equilibrated with 40% B over 2 min. Alternatively, a Grom-Sil 120 ODS-3 CP column was used for HPLC separation starting with solvent A:solvent B 60:40 at a flow rate of 0.2 ml/min. This solvent ratio was held for 3 min before a linear gradient was ranged to 100% solvent B (15 min), held at 100% B for 5 min and changed to initial conditions for re-equilibration. Samples were cooled to 10 °C in the auto sampler and the column temperature was held at 30 °C during the entire separation. Mass measurements were performed in the ESI-positive mode. The mass range from 60 to 500 *m/z* was recorded and the following parameters were applied: capillary voltage 3000 V, sample cone 10 V, source temperature 150 °C, desolvation temperature 300 °C, col-

lision energy 5 V, ion energy 1.8 V. The UV-absorbance over the range of 200–800 nm was monitored with a PDA detector. For quantification UV-absorbance at 343.5 nm was employed.

2.5. Cultivation and sampling

Specimens of *Dictyota dichotoma* and *Ulva* sp. were collected in the field close to the Station Biologique de Roscoff in France and transferred into the laboratory within 2 days. Algae were kept in artificial seawater as described above at 16 °C.

Cultures of the cosmopolitan, bloom forming coccolithophorid *E. huxleyi* (CCMP 1516) are available from the Provasoli–Guillard national centre for culture of marine phytoplankton (USA). *E. huxleyi* was grown in standing cultures at 16 °C in 100 ml artificial medium. Illumination was provided with a 14:10 h light:dark rhythm. Cells were counted with a Neubauer improved chamber (Marienfeld, Germany).

2.6. Preparation of the internal standard

DMSP*HCl and [$^2H_6$]-DMSP*HCl were synthesized according to the method of Chambers et al. [30]. Briefly, for the generation of DMSP *HCl 1 ml (14.5 mmol) of acrylic and 2.5 ml (34 mmol) DMS were dissolved in 15 ml of methylene chloride. Under stirring at room temperature excess hydrogen chloride gas was bubbled through the solution. The resulting solid precipitate was isolated by filtration and then recrystallized from methanol–diethylether (1:1). Purity was verified by ^{1}H NMR and this DMSP*HCl was used for method development. For preparation of the internal standard 2 ml (27 mmol) [$^2H_6$]-DMS was used for the reaction with 800 μl acrylic acid in 12 ml of methylene chloride as described above. Due to deuteration, purity was additionally verified by UPLC/MS and ^{13}C NMR.

2.7. Linearity, quantification and precision

Linearity and detection limits were determined for undeuterated DMSP which was derivatized using the protocol outlined in Section 2.3. Samples corresponding to 1 mg DMSP*HCl ml^{-1} methanol were diluted with methanol to reach concentrations of 0.1, 0.5, 1, 5, 10, 50, 100, 500 ng, 1 and 2 μg of the analyte per injection. They were measured by UPLC/MS and HPLC/MS as described above. Linearity was constructed by plotting the area ratios of the molecular ion (M^+) of PDAM–DMSP (349 *m/z*) relative to the DMSP-concentration. The detection limit was defined as the amount of DMSP required to reach a signal to noise value ≥10 in four repeated measurements. Intra-day and inter-day precision was determined by replicate analysis ($n = 20$) of two sets of samples spiked with 10 and 100 ng/μl DMSP within 1 day or on 2 consecutive days. For higher concentrations the area ratios in the UV spectrum (343.5 nm) relative to the DMSP-concentration could be used. To eliminate potential interference of matrix effects and hydrolysis of DMSP during the work-up of algal samples, [$^2H_6$]-DMSP as an internal standard was added after shock freezing of the samples. For quantification the amount of DMSP in the samples was determined using the area ratios of the M^+ ion (349 *m/z*) of PDAM–DMSP in the algal samples relative to the M^+ ion (355 *m/z*) of the derivatized deuterated internal standard.

2.8. Stability of the derivates

The stability of the PDAM-derivate of DMSP was monitored by injecting the same sample 0, 17, 26 and 92 h after derivatisation. During the entire experiment samples (2.93 mM) were stored at 10 °C in three different solutions (methanol, methanol + 0.01% acetic acid and methanol + 0.1% acetic acid). Stability upon prolonged storage of 90 days in MeOH was verified with samples stored at 4 °C.

To determine the stability of DMSP in marine alga samples we added [$^2H_6$]-DMSP directly to the algae and performed a one point calibration, which was compared to values using an external calibration.

2.9. Extraction of algal samples

Tissue samples of *D. dichotoma* (155–338 mg) and *Ulva* sp. (117–216 mg) were treated with the internal standard [$^2H_6$]-DMSP (500 μg/ml in MeOH) to reach a final ratio of 50 μl standard solution per 100 mg alga. These samples were shock frozen to suppress enzyme action, ground in a mortar and extracted with 1 ml methanol, 5% perchloric acid or a mix of methanol:chloroform:water (12:5:1). After centrifugation the supernatant was collected and the solvent was removed under a stream of nitrogen. The residue was taken up with 200 μl methanol. For derivatisation of DMSP an excess of PDAM solution (1 ml of a 1.2 mg/ml solution of PDAM in $CHCl_3$/MeOH (3:1)) was added to the methanol extracts and stirred at room temperature for 90 min at pH 5.0. After removal of the solvent under a stream of nitrogen, 200 μl methanol were added. Samples were then centrifuged, and submitted to UPLC/MS or HPLC/MS as described above.

Samples (150 ml) of cultures of the microalga *E. huxleyi* were concentrated by centrifugation (10 min, 6000 rpm at 13 °C) in a "HERMLE Z 383 K" centrifuge (Wehingen, Germany). Seawater was removed until a volume of 0.5 ml remained. Cells were counted and 10 μl of the internal standard [$^2H_6$]-DMSP (1 mg/ml in MeOH) were added. A Bligh and Dyer treatment using MeOH and $CHCl_3$ to reach a final ratio of MeOH/$CHCl_3$/H_2O, 12:5:1 was performed for cell lysis [31]. The solvents were removed under a stream of nitrogen, the samples were re-dissolved with 200 μl methanol, derivatized and measured as described above.

3. Results and discussion

3.1. Synthesis of DMSP and [$^2H_6$]-DMSP

DMSP was synthesized in good yields following a protocol described in [30]. Due to a low pH (<1) as a result of the reaction conditions hydrolysis is suppressed during the purification steps [30]. The product was confirmed by ^{1}H NMR which showed a

>95% purity of the synthesized DMSP *HCl (^{1}H NMR (MeOD-d_4, 400 MHz) δ: 3.55 (t, 2H); 2.99 (t, 2H); 3.01 (s, 6H)). The dry solid DMSP *HCl is stable over more than 4 weeks at −20 °C. As internal standard for LC/MS we selected [$^2H_6$]-DMSP with deuterium labels at the methyl residues, which is easily accessible following the same protocol as used for the preparation of DMSP. This standard behaves similar to the unlabelled DMSP with respect to derivatisation and extraction and allows easy and background free determination in MS due to a mass shift of the molecular ion to 355 (M^+). Moreover, the use of an isotope labelled standard allows to compensate for losses due to DMSP-lyase action, which should accept both, DMSP and its labelled analogue as substrate. Using commercially available [$^2H_6$]-DMS the degree of labelling was >98%. Purity (>90%) of [$^2H_6$]-DMSP was verified by NMR and LC/MS. ^{1}H NMR (MeOD-d_4, 400 MHz) δ: 3.55 (t, 2H); 2.99 (t, 2H); ^{13}C NMR (MeOD-d_4, 100 MHz) δ: 25.67 heptett, 29.72, 40.45, 173.51.

3.2. Derivatisation of DMSP

DMSP has no strong absorbance within the UV range and thus the use of established analytical HPLC methods risks strong interference with co-eluting impurities [27]. Moreover, the extraction and enrichment of this polar analyte is problematic. We opted to use 1-pyrenyldiazomethane (PDAM) as a derivatisation reagent for the acid moiety (Fig. 1). Esterification of organic acids with PDAM occurs readily and reliably in methanolic medium and water does not interfere with the reaction [28], which is a pre-requisite for DMSP determination out of natural sources. The product exhibits strong absorbance within the UV range with a maximum at 343.5 nm. In addition, a fluorescence detector might be used to increase the sensitivity, but this equipment was not available during this study. ESI MS-detection is supported by the positive charge of the PDAM–DMSP derivative. After derivatisation of the polar DMSP with the large, hydrophobic pyrenyl residue, the product can be extracted with organic solvents and is suited for separation on a standard reversed phase UPLC or HPLC column (Fig. 2). Using an excess of PDAM, reliable quantitative analysis can be performed as shown in QC experiments. Experimental verification showed that there was no requirement for additional removal of the excess derivatisation reagent by additional treatments, since sensitivity, carry over and detected by-products were not improved by addition of acetic acid after the derivatisation reaction (data not shown).

3.3. Method development

3.3.1. DMSP analysis

The chromatographic separation was developed for the PDAM-derivate of DMSP. For direct quantification of DMSP in algal samples calibration using an external standard can be performed or, alternatively, [$^2H_6$]-DMSP can be used as internal standard if a MS detector is available. The positively charged

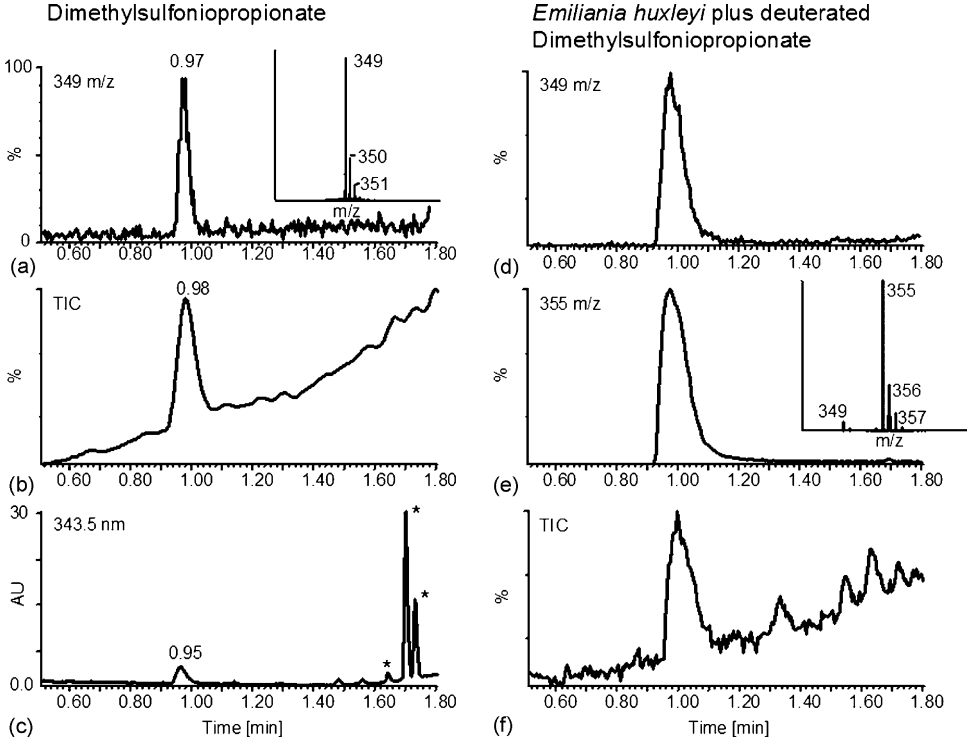

Fig. 2. Typical chromatograms (a–c) UPLC separations of PDAM–DMSP: (a) monitoring the molecular ion at 349 *m/z* (5 ng injection), the insert shows the mass spectrum of PDAM–DMSP; (b) monitoring the total ion count (100 ng injection); (c) with UV detection at 343.5 nm (100 ng injection). The signals labelled with an asterisk are also found in the control run with the derivatisation reagent; (d–f) determination of DMSP from 98.000 cells *E. huxleyi*: (d) monitoring the molecular ion at 349 *m/z*; (e) monitoring the molecular ion of the deuterated internal standard at 355 *m/z* (the insert shows the mass spectrum of [$^2H_6$]-PDAM–DMSP), (f) monitoring the total ion count. The signals at 1.34 min (*m/z* = 409); 1.53 min (*m/z* = 215), 1.61 min (*m/z* = 327) and 1.71 min (*m/z* = 301) correspond to unidentified algal metabolites.

PDAM–DMSP can be monitored in ESI-positive mode due to the M^+ ion at *m/z* 349. With increasing collision energy a dominant fragment at *m/z* 215 can be observed. The composition of the mobile phase was optimized to give best results relative to sensitivity, peak shape and separation. Water containing 0.1% acetic acid in combination with an organic solvent containing 0.1% acetic acid was used. Acetonitrile as organic solvent yielded good DMSP peak shapes but resulted in carry over effects, possibly caused by poor solubility of the derivatives in the sample syringe or loop. An alternative solvent was MeOH/tetrahydrofuran containing 0.1% acetic acid which resulted in good peak shapes with lesser carry over (Fig. 2). To monitor for carry over, a methanol injection was measured between the DMSP analyses. This procedure showed that only for samples containing more than 6 mM PDAM–DMSP detectable carry over is observed if MeOH/tetrahydrofuran is used as organic mobile phase. The same solvent system was used for HPLC separation. Compared to the HPLC method, where the PDAM–DMSP derivative eluted after 8 min UPLC separation proved to be superior with respect to run time. HPLC runs typically required a run time of 30 min (including column re-equilibration) compared to 5 min using UPLC. Detection limits in both methods were similar, around 590 nM, which is in accordance with literature findings that UPLC detection limits are in the same range or higher than those of HPLC protocols [32].

3.3.2. Linearity, detection limit and precision

Using UPLC/MS the ratio between peak areas and injected concentrations was linear over the range of 2.93 μM to 2.93 mM if the areas of the M^+ signal of the PDAM–DMSP derivative were monitored. The detection limit was at 590 nM concentrations corresponding to an injection of 1 ng PDAM–DMSP (10 μl injection volume). The typical calibration curve ($n = 4$) for the areas of the molecular ion was: $y = 544.9x + 16.75$. The correlation coefficient (r^2) was ≥0.99. Detection with UV was linear from 29.3 μM up to 11.72 mM if the area ratios of the UV signal at 343.5 nm relative to the initially applied concentration were considered. The calibration curve was: $y = 266.6 \times 10^4 x - 16.6 \times 10^4$ with a correlation coefficient (r^2) ≥0.99. The use of both detectors offers a wide linear range for DMSP detection in marine samples. Use of a fluorescence detector, which was not available during this study, would presumably extend the detection limit down to a single digit pg per injection level as reported for other carboxylic acid PDAM-derivatives [33]. This lower detection limit would then allow to directly investigate plankton samples where typical concentration ranges of 5 to >200 nM DMSP in the surface ocean are found [1,3,4]. Intra-day precision was 2.2% for 100 ng/μl samples and 3.7% for 10 ng/μl samples. Inter-day precision was 3.7% for 100ng/μl samples and 12.5% for 10 ng/μl samples.

3.3.3. Stability

PDAM–DMSP samples in different solvents (methanol, methanol + 0.01% acetic acid and methanol + 0.1% acetic acid) were monitored for their stability over 92 h at 10 °C. In the freshly derivatized samples UPLC/MS revealed only ca. 1–2% PDAM-derivatized acrylic acid, which is the hydrolysis product of PDAM–DMSP. During the 92 h under investigation a recovery of >90% of PDAM–DMSP could be found in all tested samples. Even after prolonged storage of 90 days at 4 °C in methanol recovery remained >90%.

3.4. Determination of DMSP in algal samples

3.4.1. Comparison of different extraction procedures

Several procedures were previously tested for the extraction of DMSP from sugar cane and macroalgae [27]. Plankton and microalgal samples were usually investigated indirectly using alkaline DMSP-cleavage and subsequent headspace analysis of the resulting DMS (see, e.g. [3,4]). Following established protocols for macroalgae [27], we tested three different media for extraction of DMSP from samples of the brown alga *D. dichotoma*. After freezing of algal pieces with liquid nitrogen, extractions with (a) methanol, (b) 5% perchloric acid or (c) methanol:chloroform:water (12:5:1) were performed. Procedure (a) gave highest amounts of DMSP while, in agreement with the literature [27] (b) gave lowest amounts of DMSP even if buffering of the perchloric acid was avoided during work-up. Relative extraction efficiencies were 100% for method (a), 33% for (b) and 51% for (c). The methanol extraction (a) was thus selected in this study for the investigations of macroalgae. To inactivate enzymes, three methods were tested on alga samples: (a) shock freezing with liquid nitrogen (b) boiling (c) stirring in 1 N hydrochloric acid. Procedures (b and c) resulted in very low DMSP recovery but elevated amounts of the hydrolysis product PDAM-acrylic acid were detected. Consequently, a procedure involving initial freezing of marcoalgal tissue in liquid nitrogen followed by methanol extraction of the cold material was chosen for the extraction of DMSP. Recovery rates of 50% (determined using the internal standard) were obtained, presumably due to incomplete suppression of DMSP-lyase activity.

Despite lower extraction efficiency in macroalgae, methanol:chloroform:water (12:5:1) was used for DMSP determination from microalgal cultures. This was the method of choice to at least partially suppress DMSP-lyase activity in the aqueous samples. Here, the recovery rate was 20% but quantification could nevertheless be performed reliably.

3.4.2. Determination of DMSP in macroalgae

The brown alga *D. dichotoma* and the green alga *Ulva* sp. were used for the identification and quantification of DMSP. It is well documented that *Ulva* sp. are a rich source for DMSP [11,27] which makes this alga an ideal candidate for method development. *D. dichotoma* was selected as second, lower producing species, since ecological interest in this alga is arising after the finding that DMS release might be involved in a new mechanism of activated defence (Wiesemeier, submitted for publication). Accurate mass measurements of PDAM–DMSP were conducted to prove the identity of DMSP in *D. dichotoma* (349.1260 *m/z*, calculated: 349.1257 *m/z*). In *D. dichotoma* quantification with an internal standard yielded in 71.95 ± 13.93 μg/g alga (fresh weight) DMSP, which corresponds to an amount

of 7.2‰ of the fresh weight. *Ulva* sp. contained 0.2% of the fresh weight DMSP (1.99 ± 0.17 mg/g alga DMSP). This result is in agreement with literature data for *Ulva* sp. [11,16]. The observed high variability of DMSP in algal samples urges for a more comprehensive investigation of the DMSP content of macroalgae.

Using an internal standard revealed that significant losses of DMSP during work-up cannot be avoided. Even using a protocol involving initial shock freezing of the sample and extraction with methanol the rapid enzymatic DMSP hydrolysis can presumably not be entirely prevented. This is in accordance with a pronounced DMSP-lyase activity of the investigated algae ([11], Wiesemeier, submitted for publication).

3.4.3. Determination of DMSP in microalgae

The cosmopolitan coccolithophorid *E. huxleyi* is a phytoplankton species known to contain DMSP. It is a widely used alga to study the global sulphur cycles and the role of sulphur compounds, especially DMSP, in the chemical defence [9,12,15]. After centrifugation and extraction 10.8 ± 3.3 pg/cell DMSP was identified and quantified from 100 μl of a concentrated cell suspension of 98.000 ± 10.000 cells (Fig. 2). In literature studies a high plasticity of DMSP per cell values have been observed depending on the strain investigated [12,34]. Our results are in the range observed but indicate that the strain investigated in this study is a strong producer of DMSP [12,34].

4. Conclusion

We developed a direct and efficient UPLC/MS method for DMSP determination in marine micro- and macroalgae. This method can be directly adapted for the use with standard analytical lab equipment such as HPLC coupled to fluorescence, UV or MS detectors. Through direct quantification of DMSP as its PDAM-derivate the newly developed method overcomes the problems of established procedures, which are based on the detection of the DMSP lysis product DMS. This method therefore prevents wrong determinations of DMSP which might be caused due to interference of other (biogenic) sources of DMS.

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