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Comparison of methods for DMSP measurements in dinoflagellate cultures

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Abstract

A comparison of three analytical methods (the indirect GC-FPD and MIMS, and direct LC-MS/MS) for dimethylsulfoniopropionate (DMSP) measurements was conducted to assess their accuracy and reliability. The three methods showed a linear response but are distinguished by their linearity range, the largest being for MIMS. All three methods showed good precision on *Alexandrium minutum* samples (2–12%). The variability between the three methods when comparing analyses of *A. minutum* replicates was 11%, with the DMSP measurements by LC-MS/MS being the highest. This result also confirms that indirect DMSP measurement after hydrolysis for GC or MIMS methods does not lead to an overestimation of DMSP values in *A. minutum*. A special focus was made on the more recent LC-MS/MS method including further assays in sample preparation and storage from cultures of the dinoflagellate *A. minutum*. Dinoflagellate cells should be harvested by gentle filtration (< 5 cm Hg) or slow centrifugation (500 × *g*) to retrieve the largest DMSP pool. For the LC-MS/MS method, MeOH used for cell extraction should be added prior to freezing (to prevent DMSP degradation). Samples will then be stable in frozen storage for at least 2 months. Finally, direct and indirect methods are complementary for identifying the exact DMSP fraction among dimethylsulfide-producing compounds that compose total and particulate DMSP pools issued from newly screened organisms or environmental samples.

Dimethylsulfoniopropionate (DMSP) is the biogenic precursor of dimethylsulfide (DMS), a gas involved in climate regulation and, the global sulfur cycle (Charlson et al. 1987). DMS is also a keystone metabolite in the chemical ecology of some phytoplankton cells that impact their surrounding organisms, from microbes to the upper trophic chain predators (Ferrer and Zimmer 2013; Caruana et al. 2020). The scope of research interest in DMSP is widening due to our need to understand and preserve ocean life as well as to characterize the global sulfur budget and climate change. Dinoflagellates appear to be one of the major phytoplankton groups producing DMSP (Caruana and Malin 2014) and the taxonomic group predominantly expressing the algal DMS-releasing enzyme from DMSP among eukaryotes (Shemi et al. 2023).

Since DMS has become worthy of scientific concern, several methods have been developed for DMSP measurements in seawater or in cultures of marine organisms, for use during field campaigns or in laboratory experiments. Gas chromatography (GC) was initially used to measure the DMS gas released in stoichiometric proportion by strong alkali treatment of DMSP, thus providing an indirect measurement method (White 1982). While GC coupled with a flame photometric detector (GC-FPD) was the most common method for analyzing DMSP, DMS, and DMSP lyase (DLA) (an enzyme that cleaves DMSP into DMS) in seawater and microalgae cultures (Keller et al. 1989; Steinke et al. 2000; Caruana 2010; Caruana et al. 2012), GC was occasionally coupled with various other detection systems such as pulsed FPD (PFPD), flame ionization detector, sulfur chemiluminescence detection or a mass spectrometer (MS) (Table 1) (Careri et al. 2001; Niki et al. 2004; Swan et al. 2014). Other methods have also been used on a sporadic basis for measuring DMS in seawater such as atmospheric pressure chemical ionization–mass spectrometry (Saltzman et al. 2009; McCulloch

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Additional Supporting Information may be found in the online version of this article.

Table 1. Analytical methods used for DMSP, DMS, and DMSP lyase (DLA) measurements in diverse sample types: standards only, seawater (SW), and organisms, during laboratory or fieldwork with their associated references. Methods are presented according to their category and chronological order of publication within the category, with their associated references.

Analytical methods	Sample types: standards, SW, organisms	DMS, DLA, DMSP fractions: t—total, p—particulate, d—dissolved	Lab—L, field—F	References
GC-FPD gas chromatography with flame photometric detection	Many microalgal groups including dinoflagellates	DMSPp, d, t, and DMS	L	Keller et al. (1989)
GC-FPD	SW	DMSPt, p and d	F	Belviso et al. (1993)
GC-FPD	Coccolithophores SW	DLA	L, F	Steinke et al. (2000)
GC-FPD gravity filtration	SW	DMSPp, d	F	Kiene and Slezak (2006)
GC-FPD	Dinoflagellates	DMSPp, d, t DLA	L	Caruana (2010) Caruana et al. (2012)
GC-PFPD pulsed FPD	Diatoms cyanobacteria	DMSPt, p	L	Bucciarelli et al. (2013)
GC-FPD with different columns GC-PFPD GC-SCD sulfur chemiluminescence detection PTR-MS proton-transfer reaction mass spectrometry	Standards	DMSP	L	Swan et al. (2014)
GC-FPD acidification method	Macroalgae	DMSPp	L	Bucciarelli et al. (2021)
LC-FPD (liquid chromatography with flame photometric detection)	Standards	DMSP	L	Howard and Russell (1996)
On line purge and trap-GC-FID flame ionization detection	Standards macroalgae	DMS	L	Careri et al. (2001)
GC-MS	SW	DMS	L	Smith et al. (2018)
Use of deuterated internal standards, acidification	Coccolithophores	DMSP		
SPME—GC-MS	SW	DMS, DMSPt, p, d, DLA	F	Niki et al. (2004)
PTR-MS	Coccolithophores SW	In vivo production of DMSP	L, F	Stefels et al. (2009)
PT-APCI-MS-MS	SW	DMS	F	McCulloch et al. (2020)
UPLC-MS QTOF C18 column/DMSP derivatization /internal stds	Microalgae (haptophyte) macroalgae	DMSPp	L	Wiesemeier and Pohnert (2007)
LC-MS (QTOF, HILIC) internal stds	Standards	DMSP	L	Spielmeyer and Pohnert (2010)
LC-MS with PFPP column	Coral tissues	DMSP	L	Li et al. (2010)
LC-MS/MS	Mammalian serum	DMSP	L	Lenky et al. (2012)
LC-MS	Coccolithophore dinoflagellate	DMSPp	L	Gebser and Pohnert (2013)
LC-MS and HS-GC-MS	Corals	DMSP	L, F	Swan et al. (2017)
GC-FPD and LC-MS (UHPLC, HILIC)	Bacteria	DMSPt	L	Curson et al. (2017)
GC-FPD and LC-MS (UHPLC, HILIC) extraction by acetonitrile	Bacteria microalgae including haptophytes, diatom, and dinoflagellate	DMSPp	L	Curson et al. (2018)
LC-MS/MS	Dinoflagellates	DMSPp	L	Caruana et al. (2020)
LC-MS (ZIC-HILIC, UHPLC, ESI-QTOF)	Microalgae including dinoflagellates, haptophytes, cryptophyte, and diatoms	DMSPp	L	Gebser et al. (2020)

(Continues)

Table 1. Continued

Analytical methods	Sample types: standards, SW, organisms	DMS, DLA, DMSP fractions: t—total, p—particulate, d—dissolved	Lab—L, field—F	References
MIMS membrane inlet mass spectrometry	SW	DMS	F	Tortell (2005)
MIMS	SW	DMS	F	Nemcek et al. (2008)
MIMS	Haptophyte SW	DMSPd	L, F	Barak-Gavish et al. (2018)
Capillary electrophoresis and UV detection	Macroalgae, sugarcane	DMSP	L	Zhang et al. (2005) Zhang et al. (2001)
qNMR spectroscopy	Corals	DMSP	L, F	Tapiolas et al. (2013)
Mini-CIMS: chemical ionization mass spectrometer for continuous measurements onboard ship	SW	DMS	F	Saltzman et al. (2009)
BAPI-PIMS: benzene-assisted photoionization positive ion mobility spectrometry	SW	DMS	F	Peng et al. (2020)

et al. 2020), which allows almost continuous measurement onboard ship; membrane inlet MS (MIMS) for DMS and DMSP measurements in seawater or in cultures (Tortell 2005; Barak-Gavish et al. 2018); or proton-transfer reaction MS for DMS in seawater or in vivo DMSP production (Stefels et al. 2009; McCulloch et al. 2020). However, because DMS precursors other than DMSP have been reported, including minor ones (Howard and Russell 1996; Nakamura et al. 1997; Gebser and Pohnert 2013; Gebser et al. 2020), indirect DMSP measurement after chemical cleavage to DMS may overestimate the true DMSP value. Thus, further analytical methods were developed to directly measure DMSP in marine organisms such as capillary electrophoresis with UV detection, qNMR spectroscopy or liquid chromatography LC coupled with FPD or with a MS: UPLC-MS and LC-MS/MS (Howard and Russell 1996; Zhang et al. 2001; Zhang et al. 2005; Wiesemeier and Pohnert 2007; Li et al. 2010; Tapiolas et al. 2013; Caruana et al. 2020).

Despite the number of different techniques, the availability of several of them raises questions on their equivalence, sensitivity, and accuracy and thus highlights the need to understand their respective benefits and appropriate use, particularly for measuring DMSP in phytoplankton cultures. In the case of dinoflagellate cultures, the response of the organisms to the method, particularly sample preparation, needs to be considered. In the present study, we used *Alexandrium minutum* as a dinoflagellate model for DMSP production as it is known to produce substantial DMSP concentrations per cell and per cell volume (Caruana et al. 2012; Caruana and Malin 2014; Caruana et al. 2020). This species is also particularly sensitive to turbulence and handling stress (Caruana 2010; Berdalet et al. 2011), requiring careful manipulation to maintain its cell integrity. It also contains DLA, leading to potential DMSP loss and bias in its measurement. Hence, the choice of methods should also pay attention to storage conditions that can prevent further DMSP enzymatic cleavage or degradation in the sample (Russell and Howard 1996). Overall, obtaining correct results depends on each step of sample preparation from culture sampling to chemical analyses, including sample handling, cell harvesting method and storage conditions.

In this study, we made a comparison of three analytical methods for DMSP measurements (GC-FPD, MIMS, and LC-MS/MS) applied to *A. minutum* culture samples, focusing particularly on the most recent method, LC-MS/MS. The comparison included the linearity of standards, the range of detectable standards, and the comparison and precision of DMSP concentrations in *A. minutum* samples between analytical methods. We also paid special attention to the sample preparation methods (cell harvest and storage) that may influence the DMSP results in order to determine the reliability and assess the benefits of these preparation methods.

Materials and procedures

Culture conditions and sampling

A. minutum strain RCC7037 was grown in xenic batch culture with L1 medium without silicate as this is not required for dinoflagellates. The culture was illuminated by cool light tubes over a 12 : 12 photoperiod at 100 μmol photons m^{-2} s^{-1} light intensity and kept at 17°C with manual stirring. A sole culture was sampled in an exponential phase within a 2-h interval to supply several samples of the same biological matrix for analyses by the different methods. Cell density was measured on three replicates of culture samples by means of a particle counter (Multisizer III; Beckman Coulter). Concentrations of DMSP

were measured by GC-FPD, MIMS, and LC-MS/MS analyses. The analysis of total DMSP (DMSPt = particulate and dissolved DMSP) required a direct sampling of 3 mL of culture for GC-FPD or MIMS (Caruana et al. 2012). The analysis of particulate DMSP (DMSPp) relied on a cell harvesting process to measure intracellular DMSP concentrations. This was done from a 3 mL culture sample for GC-FPD and MIMS, and from a 7 or 10 mL sample for LC-MS/MS.

Experimental design for standard and culture assays

A range of standards were prepared in duplicate and run for each of the three methods (0.015–120 nmol S or 5 nmol L^{-1}–40 μmol L^{-1} for LC-MS/MS, 0.015–480 nmol S or 5 nmol L^{-1}–160 μmol L^{-1} for GC-FPD and MIMS) to characterize their linearity and the lowest detectable standard concentration. For GC-FPD and MIMS, the preparation of standards required the dilution of an aqueous DMSP stock solution in acidified water (0.2 mol L^{-1} HCl final concentration). To correspond to culture samples potentially being in seawater (DMSPt) or ultrapure water (filter and cell pellets for DMSPp measurements), standards were prepared in artificial seawater (final salinity 35) and ultrapure water to assess any potential discrepancies in the results. For LC-MS/MS, standards were prepared in methanol. In addition, to test for the potential effect of biological matrix on DMSP measurements (Spielmeyer and Pohnert 2010), standards for LC-MS/MS analyses were also prepared in the presence of cells of *Thalassiosira weissflogii* IFR-TWE-97 (cell density = 2.4 × 10^6 cells mL^{-1}, biovolume = 2.3 × 10^9 μm^3 mL^{-1}), a diatom that does not produce DMSP.

Two experiments with *A. minutum* cultures in exponential phase were conducted to compare DMSP measurements obtained by the three analytical methods LC-MS/MS, GC-FPD, and MIMS (Fig. 1). The first experiment assessed the method of cell harvesting by centrifugation. Given that mechanical stress may lead to DMSP loss (Wolfe et al. 2002), centrifugation could offer a more suitable alternative to filtration when gravity filtration is not possible, for example, when harvesting dense phytoplanktonic cultures. A culture of *A. minutum* (42280 cells mL^{-1}) was sampled to measure DMSPp concentrations by LC-MS/MS and to assess the effect of centrifugation speed, fast (3600 × *g*, 10 min, 4°C) or slow (500 × *g*, 10 min, 4°C). To determine the most accurate DMSPp value obtained, we measured DMSPt concentrations by GC-FPD and MIMS and used their mean as a reference value to which DMSPp values were compared to see which was the closest. Each treatment was performed on triplicate culture samples.

In the second experiment, another culture of *A. minutum* (24385 cells mL^{-1}) was sampled to test several steps of the sample preparation (Fig. 1). To compare the effects of filtration with those of centrifugation as methods for harvesting dinoflagellate cells, the culture samples were either filtered through a GF/F filter using a hand vacuum pump with weak pressure < 5 cmHg (Caruana 2010; Caruana et al. 2012), or the culture sample was gently centrifuged (500 × *g*) and the cell pellet retained. These gentle filtration and centrifugation conditions were chosen to limit cell damage and potential DMSPp release. For the indirect methods GC-FPD and MIMS, the filter or cell pellet was acidified and stored as described below (section of Analytical systems and procedures). For the direct method LC-MS/MS, the storage conditions were assessed by comparing the direct freezing of the filters and cell pellets until later MeOH addition or their direct immersion in MeOH followed

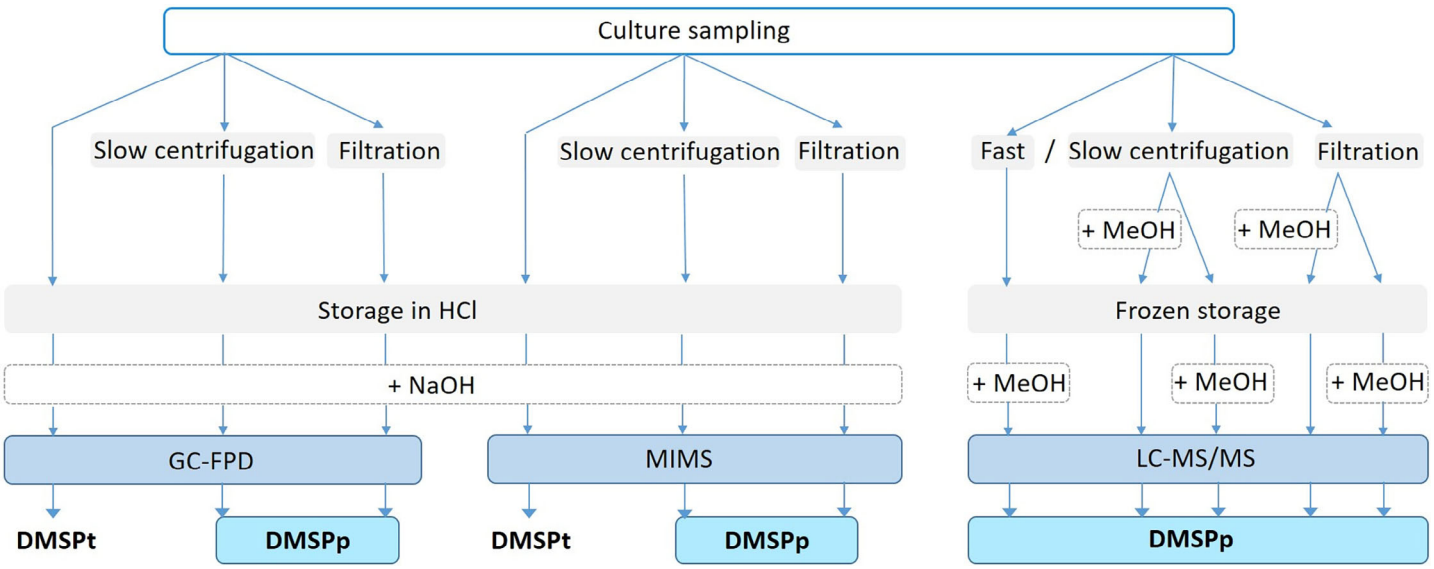

Fig. 1. Description of the different DMSP measurement methods tested, including each step from culture sampling to analysis: fast (3600 × *g*) or slow (500 × *g*) centrifugation, or filtration, addition of MeOH before or after frozen storage, GC-FPD or MIMS or LC-MS/MS analysis, total DMSP (DMSPt = particulate + dissolved DMSP) or particulate DMSP (DMSPp) measured.

by freezing (−80°C or −20°C). Measurements of DMSPt were used as reference values for the maximum particulate DMSP (DMSPp) concentration attainable with any of the three analytical methods tested. Each treatment for DMSPp or DMSPt was performed on five replicates of culture samples to allow robust comparison.

Analytical systems and procedures

GC-FPD

The 3 mL standards and samples were acidified with Normapure HCl in 10 mL vials (0.2 mol L^{-1} final concentration), sealed and stored at room temperature for 2 months (del Valle et al. 2011). A day before the analysis, the vials were opened to add 2 mL of 10 mol L^{-1} NaOH and then sealed again. The DMS resulting from the hydroxide decomposition of DMSP after 24 h was determined by direct injection of a headspace subsample into a Shimadzu 2010-Plus Gas Chromatograph. This GC was equipped with a sulfur-selective flame photometric detector (air/H_2: 70 mL min^{-1}/60 mL min^{-1}) and fitted with an equity 1 capillary column (i.d. = 0.32 mm, length = 30 m, Supelco; $T = 180$°C, carrier gas: He, 6.7 mL min^{-1}). Detector and injection port temperatures were set at 250°C. Based on our standard and sample concentrations, 40 μL samples were collected from the headspace of the vials using a gas-tight syringe and were injected directly into the GC injector port (Bucciarelli et al. 2021). Sample values were calculated based on the calibration curve ($y = ax$), the intercept being not significantly different from zero.

MIMS

The culture samples (or filters) were acidified with HCl 0.2 mol L^{-1} final concentration and stored for a couple of months in the dark at ambient temperature until further analyses. Twenty-four hours before these analyses, the culture samples were hydrolyzed with 9 mL of NaOH 2 mol L^{-1} in 12 mL Exetainers (Labco Ltd.), with the vials filled to the top to avoid any headspace and thus exclude gas exchange inside the vial. Concentrations of DMSP were determined following the MIMS analysis procedure and system thoroughly detailed in Barak-Gavish et al. (2018). Dissolved DMS concentration was quantified using a MIMS system (Kana et al. 2006) consisting of a Pfeiffer Vacuum quadrupole MS equipped with a HiCube 80 pumping station, a QME 220 Prisma Plus analyzer, and a flow-through silicone capillary membrane inlet (membrane length 25 mm; Bay Instruments). Samples were pumped from the bottom of the Exetainer tubes using a peristaltic pump (Minipuls 3; Gilson) at a rate of 1.5 mL min^{-1} through a stainless steel capillary tube and gas permeable Silastic membrane immersed in a 30°C water bath (VWR). The vacuum line integrated a cryotrap (a glass U-Tube held in a Dewar half-filled with liquid N_2). The level of liquid N_2 was regularly adjusted to maintain a temperature of −48.6 °C (± 0.2°C) (continuously checked at the bottom of the U-tube using a K-thermocouple thermometer HI 93531N Hanna). With an operating pressure after stabilization typically at 4.10^{-6} mbar, DMS was measured by scanning m/z 62 at a dwell time of 0.5 s using a secondary electron multiplier detector (SEM). Baseline drift was considered by monitoring the "zero" m/z 62 signal every hour in ultrapure water contained in a standardization flask held in the same 30°C water bath and continuously stirred to ensure complete gas equilibrium with the air. Sample values were calculated based on the calibration curve ($y = ax + b$) with "a" being the slope and "b" the intercept.

LC-MS/MS

Following the frozen storage period, samples for LC-MS/MS were immersed in MeOH if this had not been previously added, and sonicated (5 min, Bransonic Ultrasonic Cleaner 2510EDTH; Branson) to complete the cell extraction. The supernatant was filtered (0.2 μm membrane filters, Nanosep; Pall) before injection into the LC-MS/MS. The analytic system was composed of a UFLC XR system (Shimadzu) containing a Hypersil GOLD HILIC column (150 × 2.1 mm, 3 μm, ThermoScientific; Thermo Fischer Scientific) and coupled with a triple-quadrupole MS (4000 Qtrap; ABSciex). Analytical parameters were applied as previously described (Caruana et al. 2020). Sample values were calculated based on the calibration curve $y = ax + b$, and values were corrected to deduce the intercept such as $x = [(y - b)/a] + (b/a)$, resulting from the potential matrix effect in the MS.

Statistical analyses

Linear regression with the coefficient of determination R^2 were applied to characterize the linear range of the standard curves for the three analytical methods. To assess any potential discrepancies in DMSP measurements obtained by the different methods, non-parametric tests were performed using Statgraphics 18 software. For comparisons between two different methods, the Kolmogorov–Smirnov (K–S) test was applied. To compare the three methods, a Kruskal–Wallis (K–W) test was used. Differences between methods was considered significant at $p < 0.05$.

Assessment

Analytical measurements

The three analytical methods for measuring DMSP applied in the experimental conditions described here, show a good linearity of standards ($R^2 > 0.998$, Fig. 2), although over different concentration ranges. The indirect method GC-FPD displays suitable linearity between 2.1 and 240 nmol S (equivalent to 0.7–80 μmol L^{-1}). The indirect method MIMS displays broad linearity between 0.015 and 480 nmol S (5 nmol L^{-1}–160 μmol L^{-1}). It is worth noting, however, that for this method, the lowest DMSP standards (0.015–3 nmol S) are best fitted by a shallower slope than when considering all standards up to 480 nmol S (Supporting Information Fig. S1).

As in any analytical procedure, it is thus recommended to evaluate the best fit of the calibration curve in the range of the samples to be analyzed. The linearity of the direct method LC-MS/MS is in a lower and narrower range of concentrations than for the indirect methods, extending between 0.015 and 3 nmol S (or 5 nmol L^{-1}–1 μmol L^{-1}). The linearity range may be centered or extended for each method by adjusting some sampling parameters or instrument settings. For all analytical methods, the culture volume to be analyzed should be chosen to fall within a detectable range and this volume is an adjustable parameter. For LC-MS/MS, the sample extract in MeOH can be diluted to obtain the correct concentration range, hence, samples should preferably be prepared in excess concentrations to avoid undetectable concentrations. For GC-FPD analyses, the injected headspace volume can potentially be adjusted using a syringe of different volume. For instance, the DMSP concentration range presented here was obtained with a constant injected volume of 40 μL but could be enlarged with lower or higher injected volume, as long as the result remains linear. In addition, the coupling of a purge and trap system upstream of the GC-FPD would allow the detection of pmol S concentrations. The MIMS method generated the largest linearity range in our experimental conditions. However, contrary to other analytical methods, each sample is consumed by the analyses and discarded, therefore preventing technical replicates from being made on a sample. Indeed, the GC-FPD method allows successive measurements of a sample to be made during its analyses to check its repeatability, although the sample is then prone to losses and has to be discarded (unless subsamples are made before NaOH addition), while the LC-MS/MS method used only a few μL of sample and allows the sample extract to be stored for several months and used to further repeat the analyses if necessary.

The lowest detectable standard that responds linearly in the experimental conditions used here, are 0.015 nmol S (5 nmol L^{-1}) for MIMS and LC-MS/MS and 2.1 nmol S (700 nmol L^{-1}) for GC-FPD. The use of seawater or ultrapure water in the preparation of the standards has no significant effect on DMSP measurements with either the GC-FPD or MIMS method ($p > 0.05$, K–S tests for GC-FPD and MIMS; Fig. 2a,b). Otherwise, the detectable standards are the lowest for MIMS and LC-MS/MS. Nevertheless, as indicated above for GC-FPD, the limit can be linearly lowered by increasing the injected headspace volume (e.g., injecting 100 μL of headspace would allow to detect 0.8 nmol). For MIMS, the lowest standard detected here (5 nmol L^{-1}) approaches the detection limits previously reported (0.2–2 nmol L^{-1}), (Tortell 2005; Barak-Gavish et al. 2018). For LC-MS/MS, our lowest detectable standard (5 nmol L^{-1}) is much lower than the detection limit reported by Spielmeyer and Pohnert (2010) and Swan et al. (2017) (20 nmol L^{-1}, 20 μmol L^{-1}). Likewise, our linearity range (5 nmol L^{-1}–1 μmol L^{-1}) is also lower than that reported by Spielmeyer and Pohnert (2010) (60 nmol L^{-1}–50 μmol L^{-1}). The increased sensitivity obtained here likely resulted from the use of MS in tandem while the previous studies cited used an MS detection system. Nevertheless, Lenky et al. (2012) reported linearity for DMSP up to 30 μmol L^{-1} with LC-MS/MS and a detection limit of 100 nmol L^{-1}. The lowest detectable standard obtained here for GC-FPD (0.7 μmol L^{-1}) is similar to or higher than the detection limits published elsewhere such as the 0.07, 0.2–0.3,

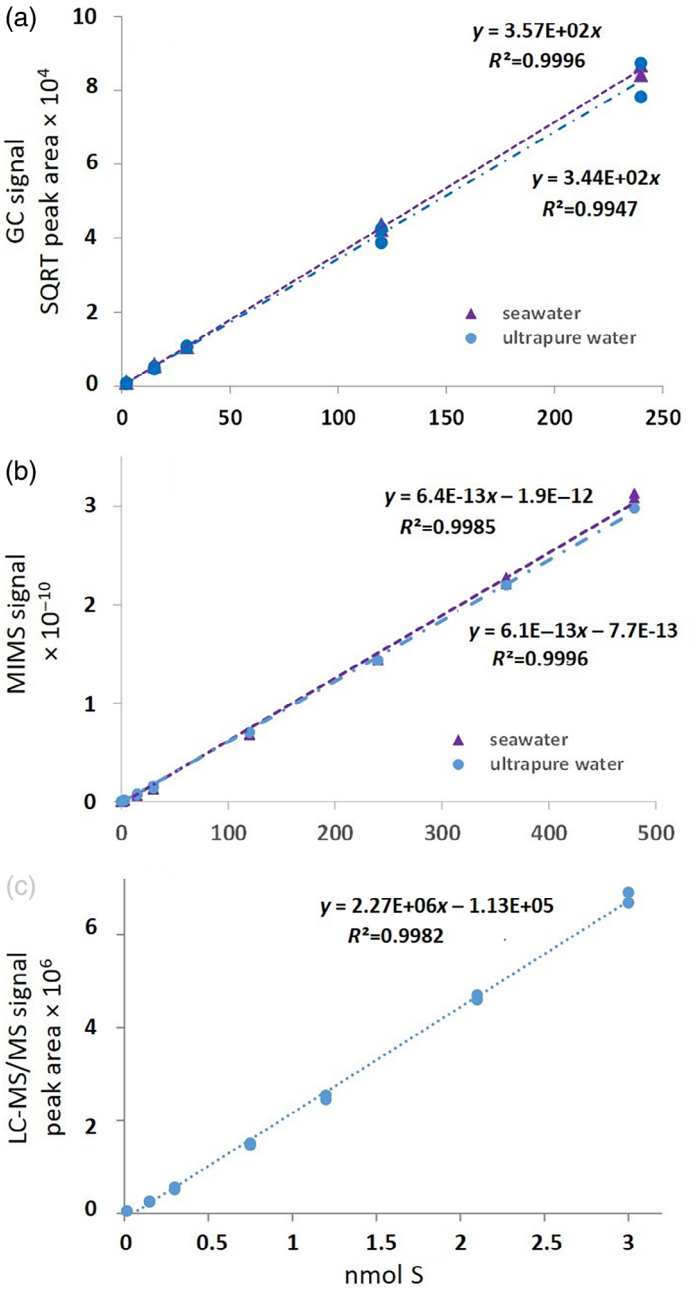

Fig. 2. Linear ranges of the standard curves obtained for each of the three analytical methods: GC-FPD (**a**), MIMS (**b**), and LC-MS/MS (**c**). For the indirect methods (**a**, **b**), two standard curves show similar results for standards diluted in artificial seawater and freshwater.

1 μmol L^{-1} reported by Caruana et al. (2012), Kiene and Slezak (2006) and Swan et al. (2014), respectively. Our calibration range is also close to those reported in a previous study by Caruana et al. (2012) (0.7–25 μmol L^{-1}) and in an inter-laboratory calibration exercise by Swan et al. (2014) (1–10 μmol L^{-1}).

For LC-MS/MS analyses, the potential effect of the biological matrix was assessed. The standard curves (50–1000 nmol L^{-1}) realized with or without microalgal cells (*T. weissflogii*) showed no significant difference ($p > 0.05$, K–S test; Supporting Information Fig. S2). The DMSP recovery in the presence of diatom cells was found to be between 90% and 95%. These recovery values may have led to a slight overestimation of DMSP measurements by LC-MS/MS, although these remain in a similar range to the intraday variation of standards (4% on average for 4 series of 7 standards over a 2–9% range). Moreover, more diatom cells were added to the standard assays than the *A. minutum* cells sampled, and the *A. minutum* sample extract was further diluted prior to LC-MS/MS measurement, thereby reducing potential biological matrix effects (Panuwet et al. 2016).

The precision of several DMSP measurements based on the coefficient of variation obtained on replicates of *A. minutum* samples ($n = 5$) was in the range of 2.5–5.0% for MIMS, 2.4% ($n = 4$) to 7.1% ($n = 5$) for GC-FPD, and 4.8–12.3% for LC-MS/MS. The precision of the methods was directly evaluated on *A. minutum* samples and therefore partly included the variation between culture samplings. The narrow range of precision obtained for all methods indicates that the whole procedure for preparing, storing and analyzing samples allows a good repeatability of DMSP measurements. Indirect methods appear to have a slightly better precision than the direct method suggesting that there could be a slight effect of the biological matrix in the direct method.

Sample collection

The accuracy of DMSP measurements in dinoflagellate cells does indeed rely on the attention paid to all steps of the method from culture sampling to analytical measurements and their improvement. For harvesting phytoplankton cells from culture or seawater samples, previous studies recommended the use of gentle filtration, taking care to keep the filter wet to avoid cell lysis in contact with air and potential DMSP loss. Gentle filtration can be achieved by gravity filtration of small-volume seawater samples (Kiene and Slezak 2006) or by using a hand vacuum pump carefully maintaining a weak pressure < 5 cmHg in the case of dense phytoplankton cultures (Caruana et al. 2012). Wolfe et al. (2002) underlined that mechanical stress, such as shaking or sparging cultures, may lead to DMSP cleavage to DMS. For cell harvesting from cultures, the filtration step may also be replaced by centrifugation. This is an advantageous method that is rapid, repeatable, and controllable in terms of temperature and speed. Reducing the temperature (4°C) of centrifugation far from its optimal temperature of 30°C, 40°C, 50°C (Yost and Mitchelmore 2012; Peng et al. 2019; Li et al. 2021), may limit enzyme activity and prevent DMSP degradation from DLAs during the process of cell harvesting.

In the first experiment with culture samples of the dinoflagellate *A. minutum*, DMSPp concentrations measured by LC-MS/MS were lower than DMSPt concentrations from indirect methods (Fig. 3). The fast centrifugation led to a reduced DMSPp concentration (53% of the mean DMSPt of the two indirect methods) compared with that obtained by slow centrifugation (80% of the mean DMSPt). These results agree with those from Wolfe et al. (2002), showing mechanical activation of DMSP cleavage, and thus imply that slow centrifugation is preferable.

In the second experiment (Fig. 4), the centrifugation step (slow) led to significantly higher DMSPp values (mean = 25.4 ± 3.36 nmol mL^{-1} for all three methods) than the filtration process using a hand vacuum pump (20.7 ± 2.34 nmol mL^{-1}) ($p < 0.001$, K–S test). The centrifugation produced a 16% higher DMSPp concentration (87% of DMSPt obtained by GC) than the filtration process (71% of DMSPt). This result potentially means, on the one hand, that the stress of gentle centrifugation is lower than that of filtration, leading to a smaller loss of DMSP. On the other hand, a higher DMSP value may have resulted from higher DMSP input due to aggregates of organic debris. The remaining bacteria do not influence DMSP measurement in exponentially growing cultures of *A. minutum* since cultures treated with antibiotics were previously shown to have similar DMSP content to untreated cultures (Geffroy 2021). It is not easy to determine which of these two techniques provides the most accurate result, however, the choice between the use of filtration or centrifugation should take into account the scientific objective (particulate, dissolved, or total DMSP) and the cell fragility or colonial or mucal aspects of the dinoflagellate species. In any case, based on our results (Figs. 3, 4), the use of slow (500 × *g*) centrifugation is recommended for harvesting dinoflagellate cells because high centrifugation pressure may cause considerable loss of DMSP, likely due to cell lysis.

Sample storage

When samples cannot be analyzed on the day of culture sampling, it is necessary to store them. Storage conditions may also impact the final DMSP concentrations detected in the sample (Russell and Howard 1996; Borges and Champenois 2017); hence, this step also requires attention for method improvement. Storage of samples harvested by filtration and intended for LC-MS/MS analyses does not appear to be affected by whether MeOH was added before or after freezing. However, the addition of MeOH before freezing to cells harvested by centrifugation significantly increases the DMSP content ($p < 0.05$, K–S test; Fig. 4). This suggests that some DMSP could have been lost as the cell pellets were removed from storage and the MeOH added. Conversely, after filtration

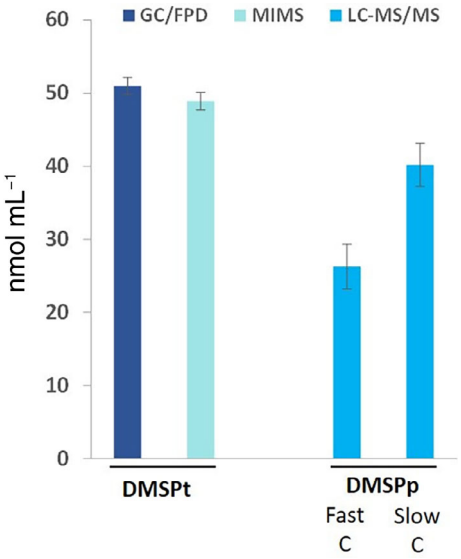

Fig. 3. Comparison between fast (3600 × *g*) and slow (500 × *g*) centrifugation (C) on the DMSP measurements obtained by LC-MS/MS and with the DMSPt concentrations measured by GC-FPD and MIMS. Error bars represent standard deviations of the means ($n = 3$ sample replicates of a sole culture).

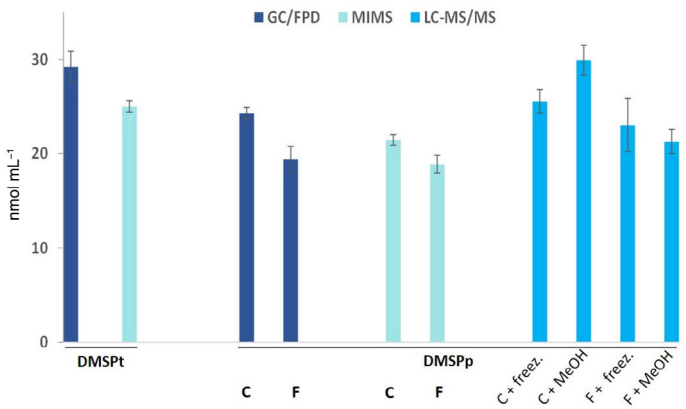

Fig. 4. DMSPt and DMSPp concentrations measured by three analytical methods (GC-FPD, MIMS, and LC-MS/MS). DMSPp measurements were obtained after either C: slow centrifugation or F: gentle filtration of the *A. minutum* sample and storage before LC-MS/MS analyses either by direct freezing (freez.) or by adding MeOH and then freezing. Error bars represent standard deviations of the mean ($n = 5$ replicate samples from a sole culture, except $n = 4$ for GC analyses after centrifugation).

and frozen storage, cells were potentially protected by the folded filter and less prone to thaw before MeOH addition. Therefore, we recommend the use of MeOH before freezing to avoid DMSP degradation during sample thawing. Indeed, in the case where MeOH is added after frozen storage, thawing needs to be prevented by carefully placing samples in ice while rapidly adding MeOH in order to avoid any DMSP degradation by cell lysis and enzymatic cleavage.

Using the LC-MS/MS method, the analyses of *A. minutum* extracts ($n = 20$) in MeOH after 2 months of storage show no significant difference compared with samples analyzed within a short-term period (2 weeks) ($p > 0.05$, K–S test; Fig. 5). Long-term storage of up to 2 months has no effect on DMSP measurements by LC-MS/MS. Frozen storage (−80°C) makes it possible to store samples in a manner that prevents any biological activity or DMSP loss, provided that MeOH is used as described above. For MIMS and GC-FPD, the addition of HCl allows samples to be stored (del Valle et al. 2011). Moreover, since our samples were only measured once after a couple of months of storage, the coherence of data obtained among the methods (Fig. 4) suggests that this acidification method is appropriate for dinoflagellate samples.

DMSPp concentrations in dinoflagellate samples obtained by three analytical methods

Comparing the DMSP data obtained by the three methods provides insights into their accuracy. In the first experiment with culture samples of the dinoflagellate *A. minutum*, DMSPt concentrations obtained by GC-FPD and MIMS were not significantly different ($p > 0.05$, K–S test; Fig. 3). These two indirect methods led to similar DMSPt concentrations even though the DMS measurement was made in the headspace for one and in the liquid sample for the other. In the second experiment, the DMSPt and DMSPp values were slightly higher when obtained by GC-FPD (29.2 ± 1.68 and 21.6 ± 2.77 nmol mL^{-1}, respectively) than by MIMS (25.0 ± 0.63 and 20.9 ± 1.55 nmol mL^{-1}, respectively); the difference only being significant for DMSPt values ($p < 0.05$, K–S test; Fig. 4). It is difficult to explain this discrepancy since the standard curves were correct and these two analytical methods gave similar results for DMSPt in the first experiment. For these indirect techniques, GC-FPD and MIMS, DMSPp concentrations represented 79.6% and 74.5% of the DMSPt concentrations, respectively. DMSPp data obtained by LC-MS/MS were significantly higher (mean = 24.9 ± 3.75 nmol mL^{-1}, $n = 20$, $p < 0.01$, K–W test) than those obtained by the indirect methods, with some values even being equivalent or higher than the DMSPt concentrations. More precisely, DMSPp values obtained by LC-MS/MS were significantly different from MIMS ($p < 0.05$, K–S test) but not from GC-FPD ($p > 0.05$, K–S test), and those from GC-FPD were not significantly different from MIMS ($p > 0.05$, K–S test). Compared with the DMSPt concentrations obtained by both indirect methods (mean = 27.1 nmol mL^{-1}), DMSPp measurements by LC-MS/MS represented 92.1% of DMSPt, thus being 18% and 12% higher than those obtained by MIMS and GC-FPD, respectively. The two latter methods also differed from LC-MS/MS by their storage procedures, which included acidification of the culture sample, while samples for LC-MS/MS were stored in MeOH. The authors del Valle et al. (2011) reported that the acidification method stabilized DMSP for months in culture samples from many phytoplankton species, with the exception of colonial haptophyte *Phaeocystis globosa*, for which samples lost 68% of their DMSP content, likely

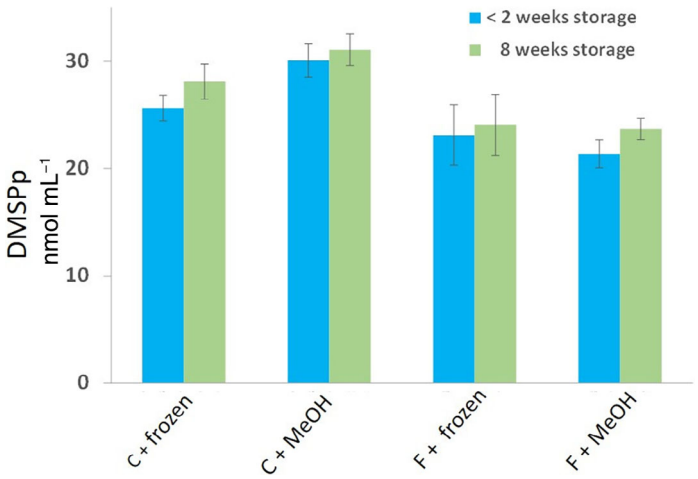

Fig. 5. Effect of 2 months of storage on DMSPp methanol extracts analyzed by LC-MS/MS. Data in blue (< 2 weeks storage) are those presented in blue in Fig. 4 (obtained for *Alexandrium minutum* samples filtered (F) or centrifuged (C) then directly frozen or frozen following the addition of MeOH).

due to DLA activity. However, this did not happen for solitary cells of *P. globosa*, or for other phytoplankton tested, including dinoflagellates and, even species with DLAs. While the species *A. minutum* may possess DLA (Caruana and Malin 2014), no lyase detection or measurement was done on the strain RCC7037 examined here. Also, even if it is unlikely that acidification could lead to DMSP loss in this strain, this cannot be excluded. Moreover, if we consider the DMSPp averages (21.6, 20.2, and 24.9 nmol mL^{-1}) for all data obtained by the three methods (GC-FPD, MIMS, and LC-MS/MS, respectively), the variability between them represented by the coefficient of variation is only 11%. In comparison, Swan et al. (2014) reported a 25% variability for a DMSP calibration between 10 laboratories that used GC methods. Moreover, the variability between DMSPp data in *A. minutum* culture was expected to be higher than for DMSP standards as it also included the variability between culture samplings and the potential effect of the biological matrix on LC-MS/MS measurements, though minimized by sample dilution (Panuwet et al. 2016). The 11% variability therefore remains acceptable, although efforts should be continued to reduce variability between analytical methods, such as by reducing the mechanical stress of culture handling for harvesting cells, evaluating the effects of the biological matrix in LC-MS/MS measurement for different phytoplankton groups or species with more precision, and developing a method for measuring the dissolved DMSP fraction by LC-MS/MS.

Discussion

Comparison of three commonly employed methods for quantitative analysis of DMSP in dinoflagellate cultures led to coherent DMSP values. The good coherence and precision of the three methods rely on all the steps of culture sampling, storage and analysis, although these differ among the methods, and the care and attention paid to these steps may contribute to explaining the low variability observed. For cell harvesting, we recommend using the gentlest means, in agreement with results from Wolfe et al. (2002) and Kiene and Slezak (2006) to prevent from cell DMSP leakage. Appropriate gentle techniques adapted to phytoplankton cultures include preferentially slow centrifugation or the use of a weak-pressure vacuum pump. Storage is also a delicate process that needs to be done in such a way as to prevent from DMSP degradation. For the LC-MS/MS method, the use of MeOH is recommended before frozen storage.

Another source of discrepancy between the methods could come from DMS precursors in the indirect DMSP methods. The LC method makes it possible to analyze DMSP directly in cell extracts, while GC-FPD and MIMS can be used to analyze the DMS that results from the chemical cleavage of DMSP. There also exist other DMS precursors than DMSP, which might cause an overestimation of DMSP values when using indirect methods. Nevertheless, DMSP seems to be the major precursor in some dinoflagellates (Caruana 2010; Gebser and Pohnert 2013; Gebser et al. 2020). *Lingulodinium polyedrum* is an exception, however, as this dinoflagellate produces major concentrations of gonyol and minor ones of DMSP, both of which are DMS precursors (Gebser et al. 2020). Our higher results with the direct method appear to indicate that indirect methods do not overestimate DMSP in *A. minutum*, suggesting that other DMSP precursors are minor or absent in this species.

MIMS is a reliable, precise, sensitive (lowest detectable standard with LC-MS/MS in the experimental conditions used here) and affordable alternative method for DMSP and DMS measurements, with a compact size suitable for an onboard laboratory during a field campaign (Table 2). However, it is a time-consuming method since each sample has to be run manually and monitored by the operator to avoid aspiration of organic debris into the capillary and record the measurement under conditions of no external gas exchange. Moreover, based on the large range of detectable concentrations, to achieve better accuracy, it is necessary to make several calibration curves for low and high concentrations. Further assays with a longer inlet membrane (75 mm length, Barak-Gavish et al. 2018) would increase molecule diffusion and could make it possible to lower the lowest detectable standard. In contrast, GC-FPD and LC-MS/MS can have automatic injection systems that facilitate and save time for their applications. GC-FPD seems to be the most robust and easiest method for DMSP measurement, also allowing DMS measurements and the lowest detection if associated with a purge and trap system. However, although the LC-MS/MS method is the most expensive in terms of the instrument, column and solvents required for the analyses, it is direct, sensitive and allows the repeatability of the analyses. Finally, a direct method is absolutely needed for identifying without doubt the presence of DMSP in newly screened organisms.

Furthermore, the three methods are complementary since direct methods (LC) are necessary for verifying the exact DMS precursor/s (whether mostly DMSP or other molecules), while indirect methods can be used to measure DMSPt, DMSPp, or DMS and rule out the potential effect of the biological matrix on DMSP measurement that might occur with LC-MS/MS equipment.

The comparison of methods aims to validate methods, potentially selecting the most accurate one, and to supply reliable data, especially in the context of the increased number of methods that have emerged in recent years (Table 1). Few inter-laboratory exercises or method comparisons have been previously conducted (Swan et al. 2014, 2017). In the field of DMSP research, reliable and precise DMSP concentrations from culture and field samples are needed, for instance, to serve as absolute values in S budgets for marine and atmospheric models or metabolic fluxes at cellular and population scales (Lana et al. 2011; Fernandez et al. 2022; Ma et al. 2022). In addition, comparison of DMSP data between studies over time and geographical locations would be made possible based on reliable and consistent data.

Comments and recommendations

The assessment of the three analytical methods clearly demonstrates that based on their linearity and precision, the three methods are valid and produce comparable values. Hence, the comparison of DMSP data from diverse studies is possible as the use of different analytical methods results in little variation in DMSP values. However, attention must be paid to sample harvesting and storage to preserve cellular DMSP content to obtain an accurate and representative measurement. For phytoplankton cultures, as it is not possible to harvest cells by gravity filtration, slow centrifugation is recommended. Direct methods such as LC-MS/MS are absolutely necessary to identify the DMSP compound in organisms but are unable to measure the gas DMS. Choice of method may depend on the needs and budget of the project (Table 2), on whether it is to be used in the laboratory or field, or on what storage conditions are possible. For example, the onboard preparation of environmental samples may be facilitated by the use of acid rather than MeOH. The choice may also depend on the DMSP range values expected, since the low DMS and DMSP concentrations in environmental samples or in cultures of low DMSP-producing species may direct the choice toward a compact and sensitive method such as MIMS or a method coupling GC-FPD with purge and trap system for sample concentration.

With oceanic DMS fluxes accounting for 70% of the sulfur natural emissions to the atmosphere (Hulswar et al. 2022), DMSP cellular levels quantification and knowledge of their variability under abiotic and biotic stresses in different

Table 2. Comparison of the detection ranges, advantages and disadvantages of the three analytical methods GC-FPD, MIMS, and LC-MS/MS.

Analytical methods	Detection ranges	Advantages	Disadvantages
GC-FPD	2.1–230 nmol S or 0.7–80 μmol L^{-1}	Coupling with automatic injection system Affordable Coupling with purge and trap system for trace DMS measurements Possibility to increase injected headspace volume	Indirect method
MIMS	0.015–480 nmol S or 5 nmol L^{-1}–160 μmol L^{-1}	Low detection limit DMS measurements possible Affordable Compact size suitable for onboard laboratory	Indirect method Time consuming due to manual sample injection and need of operator monitoring Sample fully consumed by the analysis Several calibration curves needed for low (0.015–3 nmol) and high concentrations
LC-MS /MS	0.015–3 nmol S or 5 nmol L^{-1}–1 μmol L^{-1}	Direct method allowing DMSP identification, highest DMSP yield Coupling with automatic injection system Low detection limit Use only a fraction of the sample which can be stored and analyzed several times	Expensive No possibility of DMS measurement Use of organic solvent (MeOH)

taxonomic groups are crucially needed. Further research including direct and indirect methods should examine results obtained with other dinoflagellate species, phytoplankton, macroalgae and bacterial species to determine the exact DMS source and assess the potential effect of the biological matrix on LC-MS/MS measurement.

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