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Quantification of glycine betaine, choline and trimethylamine *N*-oxide in seawater particulates: Minimisation of seawater associated ion suppression

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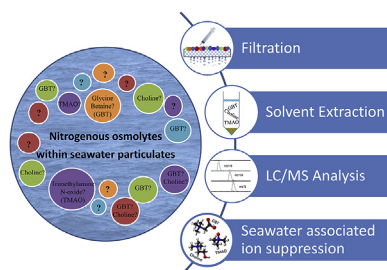
HIGHLIGHTS

- LC/MS method for measuring glycine betaine, choline and TMAO in particulates from seawater.
- The sensitivity of this method at the low nanomolar range permits its use for studies into the cycling of N-osmolytes.
- Approaches to reduce ion suppression during LC/MS of marine extracts are presented.

GRAPHICAL ABSTRACT



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ABSTRACT

A liquid chromatography/mass spectrometry (LC/MS, electrospray ionisation) method has been developed for the quantification of nitrogenous osmolytes (N-osmolytes) in the particulate fraction of natural water samples. Full method validation demonstrates the validity of the method for measuring glycine betaine (GBT), choline and trimethylamine *N*-oxide (TMAO) in particulates from seawater. Limits of detection were calculated as 3.5, 1.2 and 5.9 pg injected onto column (equivalent to 1.5, 0.6 and 3.9 nmol per litre) for GBT, choline and TMAO respectively. Precision of the method was typically 3% for both GBT and choline and 6% for TMAO. Collection of the particulate fraction of natural samples was achieved via in-line filtration. Resulting chromatography and method sensitivity was assessed and compared for the use of both glass fibre and polycarbonate filters during sample collection. Ion suppression was shown to be a significant cause of reduced instrument response to N-osmolytes and was associated with the presence of seawater in the sample matrix.

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1. Introduction

Glycine betaine (GBT), trimethylamine *N*-oxide (TMAO) and choline are nitrogen-containing osmolytes (N-osmolytes) that are widely used by organisms in the marine environment to maintain favourable osmotic tension and positive turgor [1,2]. However, other roles for N-osmolytes are beginning to be elucidated. For example, TMAO and GBT interact with photosystem I [3]. Increased recovery rates of photosystem II (PSII) have been observed in a cyanobacterium engineered to accumulate glycine betaine in the cytoplasm [4]. TMAO also stabilizes the folded state of proteins [5]. Furthermore, GBT has been shown to act as a chemoattractant in the marine microbial food web [6].

Knowledge of the distribution of nitrogenous osmolytes among

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marine phytoplankton is limited to two studies [7,8] and discrepancies exist between them. For example, Keller et al. [7] did not detect GBT in *Prorocentrum minimum*, but Spielmeyer et al. [8] found *Prorocentrum minimum* to contain the highest levels of GBT of the cultures studied. This could be due to different strains used for the two studies, different culture conditions, or methodological differences. Culture conditions have since been found to affect nitrogenous osmolyte concentrations; the production of GBT by two diatoms and a strain of *E. huxleyi* increased under both elevated temperature and carbon dioxide (CO_2) [9]. Once released from phytoplankton cells, for example by viral lysis, nitrogenous osmolytes become part of the dissolved organic nitrogen pool and are therefore an attractive substrate for marine bacteria [10]. The capacity for choline catabolism is widespread in marine heterotrophs of the marine Roseobacter clade (MRC [11]), and model organisms of the MRC can grow on choline and GBT as a sole carbon source [11] resulting in remineralisation of osmolyte nitrogen to ammonia. Similarly, MRC have been shown to use TMAO as an energy source which also resulted in ammonia production [12], and the capacity for TMAO binding in MRC is thought to be widespread [13]. Members of the Pelagibacterales bacteria (SAR11 clade) also have the capacity to degrade TMAO [14]. Marine or estuarine methanogens can also grow on nitrogenous osmolytes [15–17] indicating a link between quaternary amines and biological methane production in marine environments. Furthermore, marine metagenomic data-mining indicates the presence of genes encoding the production of trimethylamine from quaternary amines in the open ocean [18], providing a possible route and marine biogenic source of atmospheric amines [19], recently discovered to be important for new particle formation [20,21].

Despite their potential importance in the marine nitrogen cycle, particularly as a substrate for bacteria, and as potential precursors of climate-active compounds, little is known about the standing concentrations of GBT, choline and TMAO in seawater. Choline and GBT can be measured using HPLC with UV detection [22], but the method has limited sensitivity for application to natural samples. LC/MS gives much improved sensitivity for GBT and choline [23], and is a promising approach for all three analytes. Ion chromatography has been used to measure TMAO [24] in aerosol, but the sensitivity of this method is not suitable for application to seawater. TMAO has been measured previously in seawater samples off the Antarctic Peninsula following enzymatic conversion to trimethylamine [25] where it was found to be highest in surface waters, reaching 77 nM [26]. A chromatography method for choline, TMAO and glycine betaine extracted from tissues of marine fish using ion exchange chromatography has been reported previously [27], but is complex due to the use of sequential columns, and has been used to fractionate extracts for subsequent radioactive tracer determination, rather than being directly applied to quantitative analysis in seawater. A range of osmolytes from different matrices have been determined using an LC/MS approach, including mammalian serum [28–30] and coral tissues [31], but limits of detection in animal tissues and fluids are not sensitive enough for the expected concentrations in seawater [23]. Here, we present an LC/MS for the simultaneous determination of Choline, GBT and TMAO in seawater particulates.

2. Materials and methods

2.1. Chemicals

All glassware was acid-rinsed before use with 10% hydrochloric acid (purchased from Sigma Aldrich) followed by MilliQ water. Betaine hydrochloride and choline dihydrogen citrate were purchased from Sigma Aldrich. Trimethylamine N-Oxide.$2H_2O$ was obtained from Fluka. Deuterated GBT (d_{11}-GBT), used as an internal standard (ISTD), was sourced from Cambridge Isotope Laboratories Inc.. Methanol (LC/MS grade), chloroform (HPLC grade), Acetonitrile (HPLC grade), formic acid (LC/MS additive) and ammonium acetate (LC/MS grade) were purchased from Fisher Scientific.

2.2. Preparation of standards

Stock standard solutions of d_{11}-GBT (ISTD) GBT, choline and TMAO were prepared in glass volumetric flasks by weighing aliquots of the solid reference materials and diluting in methanol:chloroform:water (12:5:1). Typical stock standard concentration was 0.5 mmol per litre (mM). When not in use, standards were kept in the fridge (<4 °C). When required, stock solutions were allowed to warm to room temperature before serial dilution was performed to generate working standards over the required concentration range.

2.3. Sample collection & extraction

Seawater samples were routinely collected from Station L4, 10 km from the Plymouth coast in the Western English Channel (http://www.westernchannelobservatory.org.uk/). Surface seawater (typically 2–5 m depth) was collected aboard the *RV Plymouth Quest* in Niskin bottles attached to a rosette sampler. Seawater was transferred to a 10 L Nalgene sample bottle via Tygon tubing and transported back to the laboratory. Both the Nalgene sample bottle and Tygon tubing were pre-rinsed with seawater prior to use. The Tygon tubing was stored in 10% hydrochloric acid (HCl) when not in use, and rinsed thoroughly with MilliQ water before sampling. Transfer time back to Plymouth Marine Laboratory after sampling was typically 2 h.

Approximately 4 L of the surface seawater sample was transferred to an acid-rinsed glass beaker through a nylon mesh (pore size 200 μm to remove zooplankton), and stirred gently to homogenise cell distribution via a magnetic stirring plate. Aliquots of seawater (typically 5–100 mL) were removed via a plastic syringe and filtered through an in-line polycarbonate filter (Nucleopore; 47 mm, 0.2 μm). Before use, filters were soaked in 100% methanol (LC/MS grade) for 2 h, after which, they were rinsed in clean methanol and allowed to dry at room temperature. After filtration, the residual seawater left on the filter was minimised by blotting the underside on laboratory absorbent paper. The filter was then immersed immediately in 1.5 mL of methanol:chloroform:water (12:5:1) in a 50 mL Sarsdedt© tube. Internal standard (10 μl) was added to yield a final concentration of 10 pg per microliter (pg μL^{-1}) d_{11}-GBT. Samples were briefly vortexed and left to soak for 1 h. Samples were then re-vortexed and the solvent transferred to an Eppendorf tube for clarification by centrifugation (4 min at 13,000 rpm). Finally, the supernatant was transferred via Pasteur pipette to an autosampler vial for LC/MS analysis.

2.4. LC/MS conditions & optimisation

The LC/MS system comprised an Agilent 1200 High Pressure Liquid Chromatograph (HPLC) incorporating a degasser (G1379B), binary pump (G1367B), temperature-controlled autosampler (G1367B), and thermostatted column compartment (G1316A). The HPLC was coupled to an Agilent 6330 ion trap mass spectrometer via an Electrospray ionisation (ESI) source operated in positive ion mode.

For separation of the analytes a Discovery HS F5 column (150 × 2.1 mm, 3 μm particles) was used in combination with a guard column (HS F5 Supelguard) both supplied by Sigma Aldrich. The column temperature was maintained at 60 °C during analysis.

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