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Determination of betaine metabolites and dimethylsulfoniopropionate in coral tissues using liquid chromatography–time-of-flight mass spectrometry and stable isotope-labeled internal standards

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ABSTRACT

A convenient procedure for determination of seven betaine analogs and dimethylsulfoniopropionate (DMSP) in extracts of coral tissues using LC–MS stable isotope dilution is described. Extraction procedures were optimized for selective extraction of polar metabolites from coral tissues. The LC–MS protocol employed a pentafluorophenylpropyl (PFPP) column for HPLC separation, with chromatographic resolution of isobaric and isomeric zwitterionic metabolites optimized by adjusting the acidity of the mobile phase. A ternary gradient was used to exploit the unusual retention characteristics of cationic metabolites on the PFPP column, with incorporation of ammonium acetate in a later gradient stage promoting elution of more hydrophobic betaines which are retained at high organic content in the absence of ammonium acetate. We demonstrate that the new LC–MS based method provides accurate measurements from nanomolar to high micromolar concentrations, and can be applied for profiling of betaine metabolites and DMSP in corals or other aquatic organisms.

1. Introduction

Betaines of amino acids are quaternary ammonium metabolites and dimethylsulfoniopropionate (DMSP) is a tertiary sulfonium metabolite produced by a wide range of higher plants and marine algae, and also found in (sometimes produced by) bacteria and animals [1–3]. Each of these metabolites possesses a functional group with a permanent positive charge and an acidic moiety such as a carboxylic acid that combine to confer zwitterion characteristics at physiological pH. Betaines are reported to be non-toxic osmolytes and compatible solutes that offer protection from damaging effects of osmotic stress, drought, high salinity, high temperature or high light stress on protein and membrane functions. Betaine metabolites can accumulate to millimolar concentrations without harming the functions of cytoplasmic enzymes and other macromolecules [3,4]. In this context, accumulation of betaines to millimolar concentrations represents a potential mechanism for corals and other organisms to resist the damaging effects of stressful environments, including those with elevated temperatures. We conducted preliminary studies to explore betaine levels, in which metabolites were extracted from tissues of tridacnid (giant clams) and corals, both of which are types of organisms in which animal and algal components live in symbiosis. Mass spectra of these extracts suggested ions with masses corresponding to $[M+H]^+$ of multiple betaines (where M corresponds to the neutral zwitterionic form).

One of the challenging aspects of analysis of betaines and DMSP lies in their lack of useful chromophores, and their chemical structures have permanently charged groups that preclude gas chromatographic separation in their intact forms. In the 1980s analyses of betaines and DMSP relied on qualitative or semi-quantitative colorimetric tests that employed planar chromatography and Dragendorff's reagent [5,6]. Because these methods are limited in their sensitivity, selectivity and quantitative accuracy, and ability to resolve multiple betaines, knowledge of the identities and absolute concentrations of betaines in biological tissues remained elusive. Pyrolytic dealkylation has allowed betaines and DMSP to be detected as their dealkylated forms

Abbreviations: DMSP, dimethylsulfoniopropionate; FWHM, full width at half maximum; GABA, γ-aminobutyric acid; HPLC, high performance liquid chromatography; IC, ion chromatography; IS, internal standard; LOD, limit of detection; LLOQ, low limit of quantification; *m/z*, mass-to-charge ratio; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PFPP, pentafluorophenylpropyl silica; QC, quality control; RMS, root-mean-square; TOF, time-of-flight; UPLC, ultra performance liquid chromatography; UV, ultraviolet; XIC, extracted ion chromatogram.

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using gas chromatography [7–15]. However, these methods suffer from potential non-specificity, particularly if the dealkylated forms are also present in the native state. The reaction conditions sometimes used for dealkylation may convert other endogenous compounds to trimethylamine and dimethylsulfide after alkaline treatment. As a consequence, such methods may overestimate levels of tissue betaines and DMSP. For direct analysis of betaines or DMSP, chromophoric derivatives were developed for HPLC/UV detection [16–19]. However, during the use of this approach, the abundance of numerous other acidic metabolites compromises the selectivity of detection and analytical accuracy, and laborious extraction and sample preparation procedures are needed to remove matrix interferences. Similar derivatization schemes were developed using capillary electrophoresis and UV detection, but are subject to similar complications [20–23].

The permanent cationic moieties of betaines and DMSP make mass spectrometry an attractive method for their detection, and the advent of soft ionization techniques provides a convenient means for determining their molecular masses and for efficient and mass-selective detection [24,25]. Betaines in plant extracts were analyzed using electrospray ionization MS using off-line chromatographic clean-up [26], but the procedure employed strong basic conditions (6 M NH_3) that could cause artifacts in the measurement of metabolites such as DMSP and β-alanine betaine that are subject to base-induced decomposition. While a recent report described use of an ultraperformance liquid chromatography (UPLC)–MS method using a reversed phase column, and the procedure was applied to measure DMSP in marine algae [27], this approach relied on derivatization with a hydrophobic fluorescent tag to ensure adequate chromatographic retention on a reversed phase column and low level detection. Successful HPLC separations of betaines and DMSP have been reported using ion-exchange chromatography (IC) [17,28,29]. However, coupling of IC with MS is complicated by mobile phase salts employed for IC separations owing to suppression of electrospray ionization by salts. These earlier achievements in the application of MS have advanced the analysis of polar betaine metabolites and DMSP, but they highlight a need for improved separations of underivatized polar metabolites.

In recent years, numerous stationary phases have been developed for separations of polar metabolites [30–32]. In an earlier report from our laboratory, we described how cationic nitrogen-containing functional groups contributed to greater retention on a pentafluorophenylpropyl (PFPP) column. Such retention of cationic analytes was more pronounced at high organic composition, and was attributed to simultaneous electrostatic interactions of analytes with silanol residues and hydrophobic or polar interactions of analytes with the pentafluorophenylpropyl group [33]. Mobile phase pH was found to be a critical parameter for selectivity of separation of basic compounds on PFPP column owing to pH-dependent shifts in ionization of silanol groups and analytes [33]. Such prior research offered prospects that betaines and DMSP might be separated using HPLC on PFPP columns and coupled to a mass spectrometer for metabolite identification and quantification.

In this report, we describe a non-targeted quantitative method for measuring multiple betaines and DMSP in extracts of small biological samples without derivatization using HPLC–TOF-MS and a PFPP column for separation. This approach involves minimal sample processing, gentle processing conditions, and provides chromatographic resolution of isomeric and isobaric analytes with stable isotope-labeled internal standards for accurate quantification. In addition, we present an alternative synthetic scheme for preparation of deuterium-labeled DMSP for use as an internal quantification standard.

2. Material and methods

2.1. Chemicals

Glycine betaine hydrochloride, β-alanine, γ-aminobutyric acid (GABA), iodomethane, iodomethane-d_3, 3-mercaptopropanoic acid, and 5-aminovaleric acid were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). L-Alanine and pivalic acid were obtained from Fluka. Proline was purchased from Applied Science. Glycine, ammonium acetate, methanol, 88% formic acid, and acetonitrile were purchased from VWR Scientific, and D_2O was purchased from Isotec, Inc. Unless otherwise stated, all other reagents were analytical or HPLC grade and were used without further purification.

2.2. Collection and extraction of coral tissue samples

Samples of whole corals were collected from Curaçao (Netherlands Antilles) and frozen as soon as possible in liquid nitrogen or dry ice. Coral samples were stored in dry ice during shipping and stored in a −80 °C freezer afterward until sample extraction. Pieces of coral (~1–9 cm^2 surface area) were removed from the freezer, held at room temperature for 10–15 min, and transferred into a small polyethylene bag. Tissue was first blasted from the coral surface using a high-pressure stream of ice-cold distilled water from a Water-pik. The volume of the water with blasted tissue (collectively termed the *blastate*) was measured in a graduated cylinder. Typical volume of blastate was about 50 ml. Then the blastate was immediately mixed 2:1 (v/v) with methanol and acidified to 3% formic acid. The treated blastate was vortexed to suspend any cellular debris. A 596-μL aliquot was promptly removed, and 4.0 μL of a stock solution containing a mixture of eight deuterated internal standards was added. Based on results from preliminary coral tissue extractions, the internal standard was added to yield 60 μM (36 nmol) for deuterated betaines of glycine and proline and 6.0 μM each (3.6 nmol) for the remaining six deuterated standards. Extraction was facilitated by sonicating the extracts in an ice bath for 10 min followed by centrifugation at 8000 × *g* for 5 min and removal of the supernatant. The pellet was then resuspended in the same extraction solvent by vortexing, and centrifugation was repeated, to improve extraction efficiency. The supernatants were combined and evaporated to dryness using a SpeedVac. The dried residue was then redissolved in 600 μL of initial HPLC mobile phase (1% methanol/99% water, adjusted to pH 3.85 with formic acid) in preparation for LC–MS analysis.

2.3. Internal standard synthesis, characterization and quantification

Betaine standards that were not commercially available were synthesized following procedures described previously [34]. Betaine standards were synthesized by reaction of 1 mmol of amino acid and 16 mmol of CH_3I in 20 ml 1:1 (v:v) methanol/water mixture plus 1 g of $KHCO_3$. Reaction vessels were protected from light using aluminum foil, and reactions were allowed to proceed for 16 h at 25 °C. Deuterium-labeled betaine standards were made by incubating the same amino acids with CD_3I using the same amounts of reagents and reaction conditions. The reaction solvent was then evaporated under a stream of N_2 gas and the residue was partitioned using 5 ml of chloroform with 5 ml of distilled water. The upper aqueous layer, which contains betaines, was collected. Labeled and unlabeled standards of DMSP were synthesized by the reaction of CH_3I with 3-mercaptopropanoic acid in the same fashion but with 0.03 g of NaOH and 0.17 g of KH_2PO_4 as buffer. To stabilize the base-sensitive DMSP after solvent partitioning, the aqueous layer was promptly adjusted to pH ~ 3 by addition of

formic acid. All of the standards were stored at −20 °C until further use.

2.4. NMR characterization and quantification

Synthetic standards were characterized by ^{1}H NMR spectroscopy using a Varian Inova 300 MHz spectrometer. For synthetic standard quantification, 1.0 mg of pivalic acid was added to 1.00 ml of synthetic standard solutions and co-dried in a drying oven at room temperature. Dried residue was redissolved in 99.9% D_2O. A ^{1}H water suppression pulse was employed to suppress the water resonance to aid accurate integration of methyl proton resonances. Peak areas of the methyl group proton resonance from pivalic acid and methyl group proton resonances from betaines were integrated, and used to calculate betaine concentrations. Concentrations of synthetic deuterium-labeled betaines were determined by LC–MS by comparisons to responses from unlabeled betaines. Quantification was performed by comparing peak areas of extracted ion chromatograms of known concentrations of unlabeled betaine and DMSP standards with responses for deuterium-labeled betaine and DMSP solutions. After establishing concentrations of stock solutions of deuterium-labeled betaines and d_6-DMSP, known amounts, ranging from 1.6 to 34 nmol of deuterated betaines and d_6-DMSP, were added to each coral tissue extract, and isotope dilution was employed to quantify betaines and DMSP in these extracts.

2.5. LC–MS analyses of metabolites

Three Shimadzu LC-20AD HPLC pumps and SIL-5000 autosampler were fitted with a pentafluorophenylpropyl HPLC column (150 × 2.1 mm, 5 μm particles, Supelco Discovery-HS-F5) that was coupled to a Waters LCT Premier mass spectrometer. The column temperature was maintained at 60 °C. Separations employed a ternary gradient based on solvents (A) water, adjusted to various pH values for method optimization ranging from 3.4 to 4.3 by addition of formic acid, (B) methanol, and (C) 0.15% aqueous formic acid containing 10 mM ammonium acetate. The total flow rate was 350 μL/min and the injected volume was 10 μL. Metabolite detection was conducted using electrospray ionization (ESI) in positive mode. Nebulization gas flow was 350 L/h at 350 °C, and the source temperature was 100 °C. The capillary and the cone potentials were set to 3100 and 10 V, respectively. The mass spectrometer was operated in V optics mode, tuned to give mass resolution (FWHM) of 5000. Mass spectra were acquired at a rate of 1 s/spectrum using dynamic range enhancement, and employed a 0.05 s interscan delay. Metabolite quantification was performed by calculating extracted ion chromatograms (XICs) for $[M+H]^+$ ions for each zwitterionic betaine and DMSP using a mass window of m/z 0.2.

2.6. Preparation of calibration and quality control standards

Since no coral tissues were found to be devoid of betaines, it was concluded that determining limits of detection by spiking a blank coral tissue extract with standard betaines at low levels was not feasible. Instead, extracts of field-collected coral tissues were subjected to addition of unlabeled betaine standards at different levels. To accomplish this, calibration standard solutions were prepared by adding NMR-standardized betaine and DMSP standards into pH 3.85 formic acid solution (the initial mobile phase used for LC separation) to give eight different concentrations ranging from 0.1 to 300 μM. Aliquots of stock solutions of deuterated internal standards (IS) were added to give concentrations of 60 μM each of glycine betaine-d_9 and proline betaine-d_6, and 6.0 μM for the remaining deuterated standards. Low-, medium- and high-level quality control (QC) spiked solutions were prepared separately to contain 0.3, 20, and 250 μM of unlabeled standards and the same concentrations of deuterated standards as described above. Triplicate *M. senaria* tissue extracts were spiked with unlabeled standards to reach 20 or 250 μM of unlabeled betaines and DMSP in the extracts to serve as QC extracts.

2.7. Evaluation of analytical method performance

Peak integration, generation of calibration curves, and calculations of analyte levels were performed using the QuanLynx routine within MassLynx v. 4.1 software (Waters). Calibration curves were based upon triplicate analyses and seven non-zero metabolite levels, and linear regression of ratios of XIC peak areas of unlabeled metabolites to XIC peak areas for deuterated internal standards (A_u/A_{IS}) against levels of the unlabeled metabolites.

Limits of detection (LOD) were defined as signal-to-RMS noise of 3 for each XIC, and low limit of quantification (LLOQ) was defined as the lowest concentration that yielded signal meeting all of the following criteria: S/N ≥ 5, accuracy between 80 and 120% of the true value, and relative standard deviation ≤15%. Two different measures of LOD were calculated. Instrumental LODs were determined by adding standards to pH 3.85 aqueous formic acid and measuring S/N for different metabolite concentrations, and extrapolating to calculate the concentration for which S/N = 3. LODs for coral tissue extracts were determined by spiking extracts with deuterated internal standards, quantifying betaines and DMSP, measuring S/N ratios for each metabolite peak, and extrapolating to the metabolite concentration corresponding to S/N = 3.

Effects of the coral extract matrix on method accuracy and precision were evaluated by preparing and analyzing QC samples consisting of a coral tissue (*M. senaria*) extract with a cocktail of metabolites (20 or 250 μM) added both pre- and post-processing and three QC metabolite cocktail solutions (0.3, 20, and 250 μM for each metabolite) prepared in pH 3.85 aqueous solution. Five technical replicate analyses were performed each day for all QC solutions over a 3-day period.

To evaluate metabolite recoveries, coral tissue blastate was homogenized by vortexing and divided into three 500-μL aliquots. The same amount of unlabeled betaine cocktail (either 20 or 250 μM) was added to one aliquot before the processing steps of extraction, centrifugation, solvent evaporation, and redissolution. The same amount of betaine cocktail was added to a second aliquot after processing, and the third aliquot did not receive any unlabeled betaines. The cocktail of deuterated internal standards was added to each after processing. Recovery (R) was calculated for each metabolite using the following equation:

$$R = 1 - \frac{A_{post}/A_{IS\text{-}post} - A_{pre}/A_{IS\text{-}pre}}{A_{post}/A_{IS\text{-}post} - A_{endog}/A_{IS\text{-}endog}}$$

where A_{post} is the analyte peak area for unlabeled betaine introduced after sample processing, A_{pre} is analyte peak area spiked before sample processing, A_{endog} is peak area of endogenous analyte (no spike), and $A_{IS\text{-}post}$, $A_{IS\text{-}pre}$, and $A_{IS\text{-}endog}$ are peak areas of deuterated internal standard for the corresponding solutions. Four technical replicates were analyzed by LC–MS for each solution.

3. Results and discussion

3.1. Standard synthesis, characterization and quantification

Before our report, the standard method employed for synthesis of DMSP has been reaction of dimethylsulfide with acrylic acid [35]. A drawback of this synthetic approach is posed by the volatility and odor of dimethylsulfide. Here we developed a new synthetic method that avoids using dimethylsulfide, and allows for synthe-

sis of deuterated DMSP using the same labeled reagent (CD_3I) as was used for synthesis of deuterated betaines. Synthesis of DMSP was achieved in this case by methylation of 3-mercaptopropanoic acid using an excess of methyl iodide. The synthesis was carried out by mixing an aqueous solution of 3-mercaptopropanoic acid in pH 7.0 phosphate buffer with an equal volume of methanol, followed by addition of CH_3I. Reaction conditions avoid exposing DMSP to alkaline conditions, which promote its rapid dealkylation to dimethylsulfide and acrylate [36].

The carboxylic group on DMSP or betaines offers a secondary potential site of reaction with iodomethane to form methyl esters. To ensure that such undesirable methyl ester formation had not occurred to a significant extent, characterization of all synthetic products was performed using both LC–MS and NMR. A single peak in the extracted ion chromatogram for the protonated zwitterion was observed for each synthetic standard. NMR spectra of all synthetic standards matched expected spectra without showing detectable singlet proton resonances around 3.7 ppm corresponding to $-OCH_3$, confirming that methyl esterified products were below limits of detection ($\ll$1% of total), even in the case of the DMSP synthesis.

Separation of betaines and DMSP was achieved on the PFPP column using a ternary gradient based on water and methanol, with the aqueous pH adjusted to exploit differences in pK_a values for the carboxylic groups of the various metabolites. The more hydrophobic betaines such as 5-aminovaleric acid betaine required both a higher organic concentration and addition of 10 mM ammonium acetate to the mobile phase, owing to the strong retention of hydrophobic cationic analytes at high proportions of methanol. Using 100% methanol, 30 min of isocratic elution failed to elute 5-aminovaleric acid betaine from the column. Such retention behavior is consistent with contributions of ion-exchange mechanisms at high organic contents as observed in an earlier study [33]. Incorporation of ammonium acetate in the initial mobile phase decreased retention and resolution of early-eluting betaines, therefore this additive was added later in the gradient.

3.2. Extraction of metabolites from coral

Several extraction methods were tested on corals before selecting the method described above. Overnight extractions of pieces of coral skeleton with tissue were judged to extract metabolites from deep in the coral skeleton, and thus were presumed to sample residues left from dead tissues. Repeated extractions of whole coral pieces with water/methanol mixtures suggested a slow and persistent release of betaines from the coral skeleton or dead tissues within the skeleton, with levels in the third extraction declining to about 25% of betaine relative to the first extraction. To focus our analyses on tissues alive at the time of coral harvesting, we employed the Water-pik approach, commonly used in coral studies, for selective removal of coral tissues from the skeleton surface to minimize contributions from the bulk coral skeleton and to generate information about betaine levels in tissues alive at the time of sample collection [37]. Various mixtures of methanol and water were tested as potential blasting solutions, with methanol proving to be incompatible with the Water-pik construction. Cold water blastate was immediately mixed with methanol and acidified with formic acid to quench metabolism and minimize metabolite degradation because although most betaines are stable during extraction, DMSP and β-alanine betaine are susceptible to decomposition to acrylate under basic conditions [36,38].

The recoveries for all target betaines and DMSP from coral tissue extracts are summarized in Table 1. Since most betaines exhibit chemical stability and high aqueous solubility, the double extraction gives high recovery for analytes from 82 to 104%. The minor losses of DMSP (82–92% recovery) during sample processing do not present problems for quantitative analysis, but highlight the value of using isotope-labeled DMSP as an internal standard.

Table 1
Mean recoveries of betaines and DMSP in spiked coral tissue extracts (n = 4).

Compound	Recovery (%)	
	Low spike (20 μM)	High spike (250 μM)
Glycine betaine	95	95
Alanine betaine	90	88
β-Alanine betaine	104	91
DMSP	82	92
GABA betaine	98	91
Proline betaine	90	94
Hydroxyproline betaine	88	87
Aminovaleric acid betaine	102	94

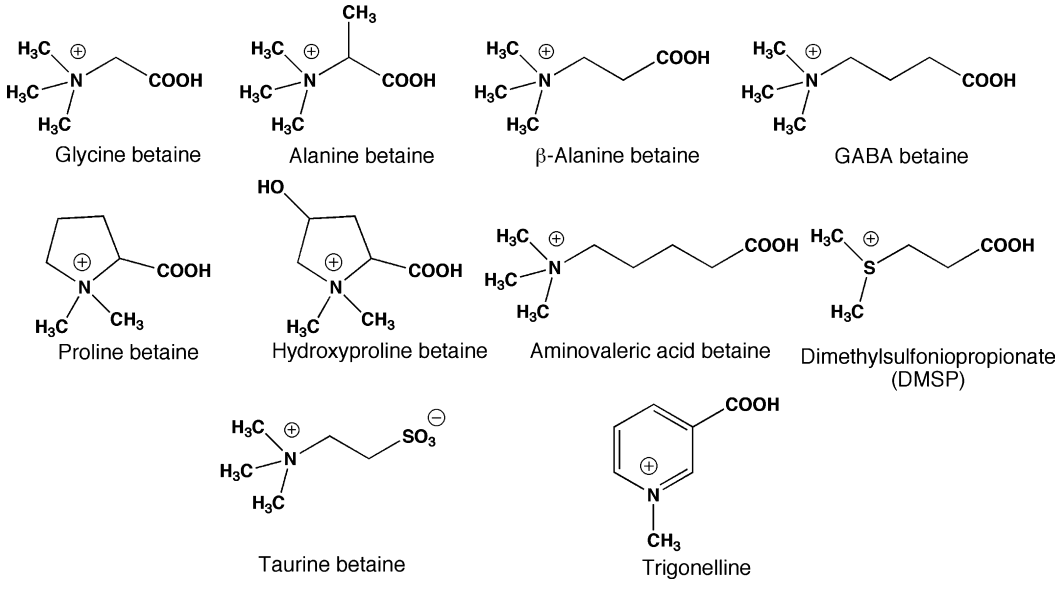

Fig. 1. Structures of betaines and DMSP detected in extracts of coral tissues.

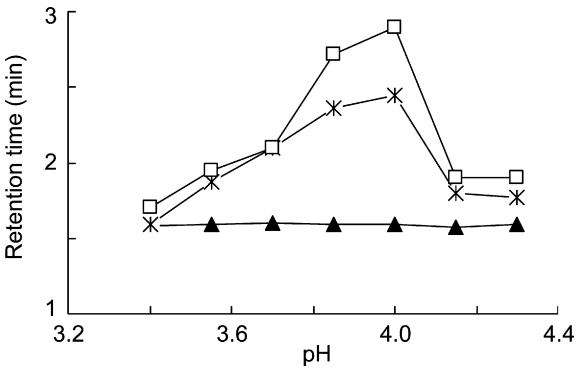

Fig. 2. Retention times of alanine betaine (□), DMSP (*) and β-alanine betaine (▲) on a pentafluoropropyl HPLC column over pH 3.4–4.3 in the aqueous mobile phase component.

3.3. LC–MS analyses of betaines and DMSP

Initial efforts to analyze betaines using an assortment of reversed phase HPLC columns (C4 and C18) and mobile phases provided unsatisfactory results owing to limited retention using all conditions. While addition of perfluoroheptanoic acid as an ion-pairing agent afforded acceptable retention, we chose to pursue separations that would not risk long-term instrument contamination by ion-pairing reagents.

As an alternative, we exploited both ion-exchange and hydrophobic retention mechanisms offered by the PFPP stationary phase, and capitalized on subtle differences in pK_a of carboxylic acid moiety to achieve separation of metabolite isomers and isobaric analytes. By adjusting the pH of mobile phase, analytes including internal standards were resolved, allowing separation of multiple betaines and DMSP metabolites (Fig. 1) in coral samples, their detection using LC–MS, and quantification by stable isotope dilution. Betaines and DMSP have groups with a permanent positive charge that confer increased water solubility and yield poor retention using reversed phase LC separations. Though retention of such metabolites can often be achieved using anionic ion-pairing reagents, such reagents often suppress ionization in mass spectrometry. PFPP columns offer enhanced retention of cationic analytes using water-organic mobile phases. Unlike alkyl silica phases that minimize ionic mechanisms of retention [39], PFPP columns exploit a combination of ionic and hydrophobic retention mechanisms that provide suitable retention of betaines and DMSP.

All tested analytes yielded abundant $[M+H]^+$ ions (based on M corresponding to the neutral zwitterionic form), but several analytes are isomers or isobaric and require chromatographic separation. In addition to the two isomers of alanine betaine, three deuterium-labeled standards all give signals at a common nominal mass: namely, alanine betaine-d_9, β-alanine betaine-d_9, DMSP-d_6 are all detected as $[M+H]^+$ at m/z 141. Subtle differences in charge and solubility were exploited to resolve these compounds (Fig. 2). Both alanine betaine and β-alanine betaine eluted in mobile phases containing <50% methanol, which corresponds to "reverse phase"-like conditions using PFPP columns [33]. Systematic adjustment of the aqueous solvent pH to a point near the reported pK_a values, yielded chromatographic resolution that could not be achieved through hydrophobic interactions alone [39]. We anticipated that the greater distance between the carboxylic group and the electron-withdrawing positive charge in β-alanine betaine and DMSP would make their carboxylic acid groups less acidic than betaines of alpha amino acids, and therefore have a higher proportion in neutral form within specific pH conditions. Adjusting pH of the aqueous mobile phase component to lie between estimated carboxylic acid pK_a values of alanine betaine, β-alanine betaine, and DMSP enhanced retention differences. Since we were unaware of reference pK_a values for all three compounds, the optimal pH for resolving these metabolites was determined empirically. Retention of alanine betaine was unchanged from pH 3.4 to 4.3 (Fig. 2). We estimate carboxylic acid pK_a values of ~2 for betaines of α-amino acids, so the specified retention behavior of alanine betaine is consistent with the carboxylic acid group being largely in anionic form at pH 3.4–4.3. For DMSP and β-alanine betaine, the greater distances between the cation and carboxylic acid groups are expected to result in less acidic carboxylic acid groups. As mobile phase pH increases, an increase in retention of both β-alanine betaine and DMSP is observed, but retention decreases upon further pH increase (Fig. 2). We attribute the increased retention to an increase in ionization of surface silanol groups, followed by a decrease in retention as the carboxylic group becomes ionized at pH >4. We reason that the optimized retention is consistent with a hydrophobically assisted ion-exchange mechanism [40,41], in which both ion–ion interaction and hydrophobic interaction are important for the retention. Mobile phase pH should be regarded as the critical parameter that can be adjusted to optimize separation of these three compounds. Optimal separation of these three metabolites was achieved at pH 3.85 for the aqueous component of the mobile phase. DMSP, alanine betaine and β-alanine betaine were well resolved using this solvent composition (Fig. 3). Adjusting column temperatures over the range of 30–60 °C had negligible effect on resolution of these three analytes.

Table 2
Solvent composition used in the ternary gradient for elution of long-chain and short-chain betaines on the pentafluorophenylpropyl silica column. Solvent A: water adjusted to pH 3.85 with formic acid, solvent B: methanol, solvent C: 0.15% aqueous formic acid + 10 mM ammonium acetate.

Time (min)	A%	B%	C%
1.00	99	1	0
3.00	99	1	0
3.01	75	25	0
6.50	50	50	0
7.50	50	50	0
7.51	0	50	50
11.00	0	50	50
11.01	99	1	0
15.00	99	1	0

Binary gradients based on aqueous formic acid (pH 3.85) and methanol failed to elute aminovaleric acid betaine, which is more hydrophobic than the other detected metabolites. Such behavior is attributed to the greater hydrophobicity and the cation-exchange retention, which is more pronounced at high organic mobile phase content. To elute this metabolite without compromising the separation described above, a ternary gradient was implemented to elute long-chain and short-chain betaines in a single HPLC separation (Table 2). This gradient was based on incorporation of a second aqueous component, after elution of the more hydrophilic betaines, that incorporated 10 mM ammonium acetate added to water, with subsequent adjustment to pH 3.85 with formic acid. The ammonium acetate provides a mechanism for displacement of the ionic retention of aminovaleric acid betaine by ammonium cation. This gradient allowed for elution and resolution of all of the betaine analytes using a 15-min gradient.

Detection of betaines and other metabolites employed time-of-flight mass spectrometry with high mass accuracy and high mass resolution. Narrow-mass-window (m/z 0.2) extracted ion chromatograms filtered out signal from ions with the same nominal mass, and yielded clean chromatograms for coral extracts that were useful for metabolite quantification. This approach aided non-targeted metabolite profiling for corals and other marine organisms, providing accurate mass measurements for metabolite discovery with simultaneous quantification of targeted metabolites

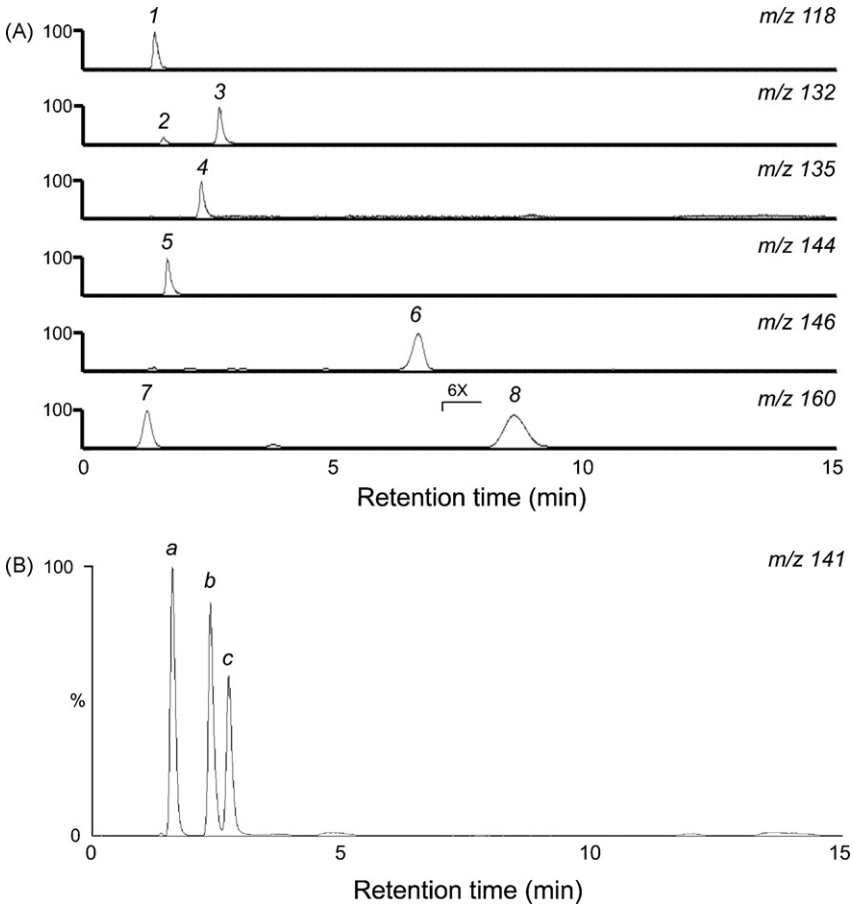

Fig. 3. Extracted ion chromatograms showing betaines and DMSP extracted from the tissue covering about 0.05 mm^2 area of skeleton in a specimen of the coral species *Madracis mirabilis* collected in the Netherlands Antilles. (A) Peak #1, glycine betaine (m/z 118, t_R 1.41 min); peak #2: alanine betaine (m/z 132, t_R 1.60 min); peak #3: β-alanine betaine (m/z 132, t_R 2.71 min); peak #4: DMSP (m/z 135, t_R 2.36 min); peak #5: proline betaine (m/z 144, t_R 1.67 min); peak #6: GABA betaine (m/z 146, t_R 6.68 min); peak #7: hydroxyproline betaine (m/z 160, t_R 1.28 min) and peak #8: aminovaleric acid betaine (m/z 160, t_R 8.57 min); (B) extracted ion chromatogram for m/z 141, which shows peaks corresponding to chromatographically resolved internal standards alanine betaine-d_9 (peak a) DMSP-d_6 (peak b) and β-alanine betaine-d_9 (peak c).

using stable isotope dilution. In addition to the anticipated betaines and DMSP metabolites described above, additional metabolites (Fig. 1) taurine betaine (observed m/z 168.0727, theoretical m/z 168.0689) and trigonelline (observed m/z 138.0555, theoretical m/z 138.0584) were observed in coral extracts and confirmed based on accurate mass measurements and separate off-line MS–MS analyses (not shown).

3.4. Method performance and validation

Although there are clear and comprehensive guidelines for validating quantitative methods in the context of administration of exogenous xenobiotics, there are far more questions than answers regarding how to validate methods for endogenous metabolites, especially for materials other than human plasma samples [42]. These challenges are particularly acute for studies of field-collected marine invertebrates because tissue availability is limited by the remoteness of sampling locations. Furthermore, the evidence from this study suggests that endogenous betaine levels are high in all coral samples collected, and no blank matrix was available that is free of betaines that could be used for spiking experiments to determine limits of detection.

To address our desire to evaluate method performance, calibration curves were constructed using 7 different concentrations for each betaine and DMSP in aqueous solution instead of sample matrices. Linear responses ($r^2 \geq 0.99$) were obtained over metabolite concentrations ranging from 100 nM to 300 μM. These curves were used to estimate analytical limits of detection for standards prepared in aqueous formic acid (pH 3.85) and using spiked coral tissue extracts (Table 3). Based on this approach, we estimate that LODs for all tested betaines and DMSP ranged from 6 to 50 nM in redissolved extracts of coral tissue, which corresponds to endogenous metabolite concentrations measured in <0.05 mm^2 of sample tissue using the current protocol. The lowest standard concentrations used in this study (100 nM) yielded accuracy and precision for all betaines and DMSP exceeding the definition of LLOQ. As a result, we conservatively estimate the LLOQ to be 100 nM for each metabolite.

Method intra-day and inter-day accuracy and precision are summarized in Table 4. All of the quality control analyses met the criteria that accuracy between 80 and 120% of the true value, and

Table 3
Limits of detection (LODs) of betaines and DMSP determined in aqueous formic acid and in coral tissue extract matrix.

Compound	LOD (nM) (in aqueous solvent)	LOD (nM) (coral tissue extract)
Glycine betaine	30	20
Alanine betaine	30	30
β-Alanine betaine	50	40
DMSP	8	15
GABA betaine	6	12
Proline betaine	30	30
Hydroxyproline betaine	8	6
Aminovaleric acid betaine	6	11

Table 4
Accuracy and precision of quality control samples with addition of multiple betaine metabolites and DMSP.

Compound	Nominal concentration (μM)	Intra-day (n = 5)		Intra-day (n = 3)	
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Glycine betaine	0.3	89	6	92	5
	20	93	4	91	5
	250	95	4	96	7
	20 (coral matrix)	106	6	103	6
	250 (coral matrix)	103	2	106	3
Alanine betaine	0.3	91	5	95	4
	20	106	6	106	3
	250	98	4	95	5
	20	91	6	94	5
	250	94	9	97	6
β-Alanine betaine	0.3	116	8	112	8
	20	98	5	95	8
	250	97	5	96	5
	20 (coral matrix)	93	3	92	2
	250 (coral matrix)	109	13	106	12
DMSP	0.3	84	3	90	6
	20	89	6	93	7
	250	95	4	96	6
	20 (coral matrix)	95	5	92	5
	250 (coral matrix)	97	5	97	7
GABA betaine	0.3	94	5	91	7
	20	106	7	112	4
	250	102	5	108	5
	20 (coral matrix)	97	7	95	10
	250 (coral matrix)	93	10	91	12
Proline betaine	0.3	91	7	93	7
	20	94	5	93	4
	250	97	4	99	6
	20 (coral matrix)	108	8	107	3
	250 (coral matrix)	108	4	105	6
Hydroxyproline betaine	0.3	90	4	91	6
	20	96	4	93	4
	250	106	7	108	8
	20 (coral matrix)	98	3	94	4
	250 (coral matrix)	103	6	99	6
Aminovaleric acid betaine	0.3	108	9	109	11
	20	96	7	95	6
	250	96	4	94	6
	20 (coral matrix)	92	9	93	10
	250 (coral matrix)	102	4	94	5

relative standard deviation ≤15%, demonstrating that the method is robust and reproducible. The data from the two quality controls made in coral extracts suggests that using the aqueous calibration curve with stable isotopic labeled standards can accurately and precisely quantify the sample in coral matrix.

All coral tissue extracts contained one or more abundant betaines, and blank field-collected coral tissues were not available for evaluating method performance using procedures normally employed for exogenous xenobiotics, as commonly practiced in a pharmaceutical analysis context. To address effects of the coral tissue extract matrix on instrument response and quantitative performance of the method, the matrix effect (ME) was calculated as

$$\mathrm{ME} = \frac{A_{post} - A_{endog}}{A_{sspike}}$$

where A_{post} is metabolite peak area for post-process standard addition, A_{endog} is the peak area of endogenous metabolite without spike, and A_{sspike} is metabolite peak area for spiked aqueous solvent without tissue extract. After addition of a betaine cocktail to a coral tissue extract at two different metabolite levels (20 and 250 μM), the processed extracts were analyzed and the effect of

Table 5
Experimental determination of matrix effect on determination of individual betaines and DMSP in coral tissue extracts (n = 3).

Compound	QC sample (20 μM)		QC sample (250 μM)	
	Matrix effect (%)	CV (%)	Matrix effect (%)	CV (%)
Glycine betaine	89	6	90	8
Alanine betaine	90	4	92	6
β-Alanine betaine	93	3	92	7
DMSP	93	7	94	8
GABA betaine	95	5	94	7
Proline betaine	93	4	93	7
Hydroxyproline betaine	93	5	94	5
Aminovaleric acid betaine	97	5	96	6

matrix on analyte signal was calculated (Table 5). The decrease in analyte signal caused by the matrix was never greater than 11% for any of the metabolites. We attribute this surprisingly small matrix effect to the high betaine concentrations found in corals, which allowed blasting of tissue quantities of a few milligrams or less into a blastate volume of about 50 ml. This extensive dilution reduces concentrations of matrix constituents, including betaines and DMSP, by 2–3 orders of magnitude. Substances that might suppress ionization are also diluted, and have minimal effects on ionization of betaines and DMSP.

Since subtle changes in mobile phase pH have critical effects on chromatographic resolution of the two alanine betaine isomers and DMSP, the stability of freshly prepared mobile phase solvent was also tested. Three replicate 250 ml volumes of solvent A were prepared at pH 3.85 and stored in 500-ml sealed glass bottles at room temperature. The bottles were opened to the air every 12 h, and the solution pH was measured using a calibrated pH meter. The rate of pH drift was calculated to be 0.02 ± 0.01 pH units/24 h, and reached pH 3.97 ± 0.03 after 7 days storage. Since baseline chromatographic resolution of DMSP and the two alanine betaine isomers was achieved over the pH range of 3.8–4, we recommend that fresh mobile phase solution A be prepared at least once a week.

4. Conclusions

The pentafluorophenylpropyl HPLC separation provides fast and effective analyses of betaines and DMSP with high accuracy typical of isotope dilution quantitative methods. Mass measurements accurate to within a few ppm help assignments of metabolite elemental formulas. Our protocol avoids analyte derivatization and employs an approach that is rapid, easy to conduct, and yields accurate and reproducible metabolite measurements. This is beneficial for analysis of unstable metabolites such as DMSP, and is suitable for analysis of field-collected tissues that are frozen upon collection. We have successfully employed this approach for quantification of betaines extracted from coral and clam tissues, and find the protocol appropriate for future investigations of metabolic responses of aquatic organisms to environmental change.

For analyses of coral extracts, the method routinely detected multiple betaines at levels corresponding to extraction of as little as 0.05 mm^2 area of tissue. The detection limits achievable (below 100 nM in extracts) highlight the potential for using this method to perform spatially resolved profiling analyses of betaine metabolites in coral tissue if suitable microsampling methods can be developed.

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