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Measurement of marine osmolytes in mammalian serum by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Osmolytes are accumulated intracellularly to offset the effects of osmotic stress and protect cellular proteins against denaturation. Because different taxa accumulate different osmolytes, they can also be used as "dietary biomarkers" to study foraging. Potential osmolyte biomarkers include glycine betaine, trimethylamine *N*-oxide (TMAO), homarine, dimethylsulfoniopropionate (DMSP), and the osmolyte analog arsenobetaine (AsB). We present a liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for the simultaneous measurement of these osmolytes in serum or plasma. Varying concentrations of osmolytes were added to serum and samples and extracted in 90% acetonitrile and 10% methanol containing 10 μM deuterated internal standards (D_9-glycine betaine, D_9-trimethylamine-*N*-oxide, $^{13}C_2$-arsenobetaine, D_6-DMSP, and D_4-homarine). Analytes were separated on a normal-phase modified silica column and detected using isotope dilution tandem mass spectrometry in multiple reaction monitoring (MRM) mode. The assay was linear for all six compounds (r^2 values = 0.983–0.996). Recoveries were greater than 85%, and precision for within-batch coefficients of variation (CVs) were less than 8.2% and between-batch CVs were less than 6.1%. Limits of detection ranged from 0.02 to 0.12 μmol/L. LC–MS/MS is a simple method with high throughput for measuring low levels of osmolytes that are often present in biological samples.

Organisms accumulate organic osmolytes in their cells and tissues to offset the adverse effects of osmotic stress. There is considerable variation in the osmolytes used across taxa [1]. Osmolytes, which include compounds such as methylamines, amino acids, sugars, and polyols, are used to protect against freezing damage, dehydration, high salinity, and pressure [2–4]. A largely unexplored application of osmolytes is their use as dietary biomarkers to identify components of the diet. Because they are often present in relatively high concentrations (millimolar or higher) in the prey tissues, osmolytes should often be measurable in the blood of the consumer after feeding. Osmolytes that we have identified as potential biomarkers are glycine betaine, trimethylamine *N*-oxide (TMAO),[1] homarine, and dimethylsulfoniopropionate (DMSP) (Fig. 1). The osmolyte analog, arsenobetaine (AsB), is also accumulated in tissues by the transport systems that transport glycine betaine but is not metabolized; therefore, it too is a potential biomarker [5].

Glycine betaine, a major methylamine osmolyte, plays an important role as an osmoprotectant in a variety of animals, plants, and bacteria [2]. TMAO acts as a protein stabilizer and is common in muscle tissues of cartilaginous and cold-water teleost fish to counteract the adverse effects of urea [6,7]. AsB, an arsenic analog of glycine betaine, is present in nearly all marine fish and crustaceans [8,9]. It has also been found to be the dominant form of arsenic in tissues of marine mammals and seabirds [10,11]. Homarine is a polar nitrogen-containing compound found in numerous marine invertebrates such as mollusks [12] and marine shrimps [13,14]. DMSP, a sulfonium analog of betaine, is produced by marine phytoplankton and algae and is accumulated by filter feeders [15–17].

Elevated levels of TMAO and AsB have been identified in the blood plasma of the Antarctic Weddell seal (*Leptonychotes weddellii*) to indicate feeding or fasting [5]. TMAO, AsB, DMSP, and homarine are not synthesized endogenously by mammals. Although glycine betaine is synthesized and metabolized in mammals [18], a dose-dependent increase in blood concentrations can be expected when an animal is feeding [19]. TMAO and AsB were previously measured separately using headspace gas

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[1] *Abbreviations used:* TMAO, trimethylamine *N*-oxide; DMSP, dimethylsulfoniopropionate; AsB, arsenobetaine; LC–MS/MS, liquid chromatography–tandem mass spectrometry; HPLC, high-performance liquid chromatography; MRM, multiple reaction monitoring; CV, coefficient of variation; LOD, limit of detection; BHMT, betaine-homocysteine methyltransferase; DMS, dimethylsulfide.

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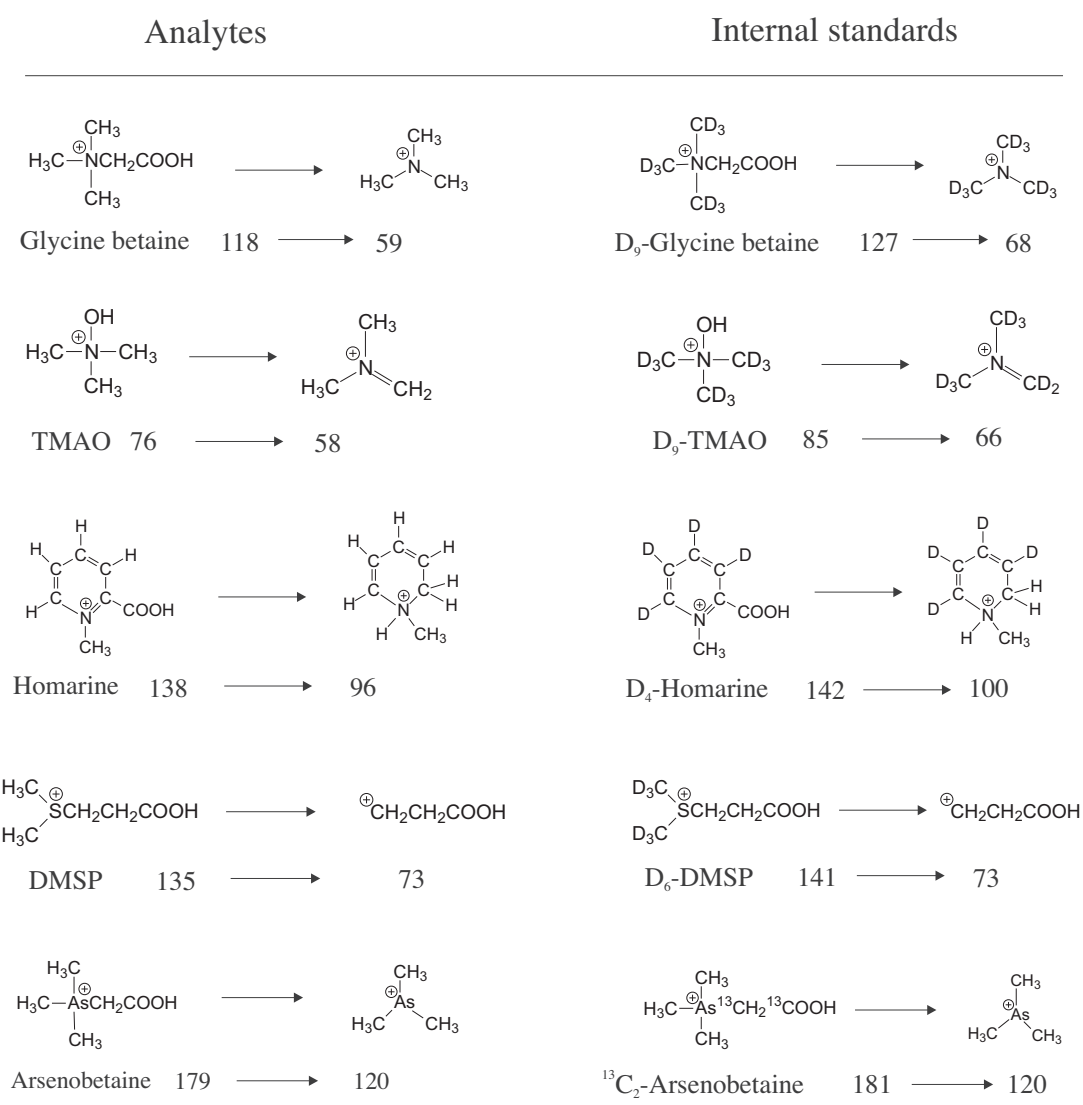

Fig.1. Mass transitions from the parent ion to the fragment ion used for detection in multiple reaction monitoring chromatograms.

chromatography (GC) and graphite furnace–atomic absorption spectrometry (GF–AAS) [5]. The aim of our study was to develop a method that could be used to identify and quantify all of the osmolytes of interest in a single sample using one analytical system.

In this article, we describe a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the simultaneous measurement of glycine betaine, TMAO, AsB, DMSP, and homarine in serum based on the method of Holm and coworkers [20]. Betaine, TMAO, and DMSP have been measured previously using LC–MS/MS [21–23]. One of the advantages of LC–MS/MS is that it has higher sensitivity than nuclear magnetic resonance (NMR) spectroscopy, so it is possible to measure the low levels of osmolytes that are often present in biological samples. LC–MS/MS also has better resolution than high-performance liquid chromatography (HPLC) techniques requiring precolumn derivatization, and sample preparation is also simpler.

Materials and methods

Reagents

Glycine betaine HCl, TMAO, and AsB were obtained from Sigma–Aldrich (St. Louis, MO, USA). DMSP was synthesized by the method of Samuelsson and coworkers [24], and homarine was synthesized from picolinic acid following the method of Conforth and Henry [25]. D_9-Glycine HCl (D_9-glycine betaine) was obtained from Isotec (Miamisburg, OH, USA), and D_9-TMAO and $^{13}C_2$-arsenobetaine were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Because D_6-DMSP and D_4-homarine were not commercially available, they required custom synthesis. D_6-DMSP was synthesized as above except that D_6-dimethylsulfide was used to react with acrylic acid. D_4-Homarine was made by reacting D_4-picolinic acid (CDN Isotopes, Quebec, Canada) with iodomethane [25]. Ammonium formate and formic acid were purchased from Sigma–Aldrich. HPLC-grade acetonitrile was obtained from Mallinckrodt (Paris, KY, USA), and methanol was obtained from Merck (Darmstadt, Germany). Bovine calf serum was obtained from ICP Biologicals (Auckland, New Zealand). Weddell seal plasma was provided by Regina Eisert from the Smithsonian Environmental Research Center (Edgewater, MD, USA). Bovine serum and seal plasma were stored at −80 °C prior to analysis.

Sample preparation

Bovine serum or seal plasma (50 μl) was added to 500 μl of extraction solvent, which consisted of 90% acetonitrile and 10%

methanol containing 10 μM of each of the internal standards (D_9-glycine betaine, D_9-TMAO, D_4-homarine, D_6-DMSP, and $^{13}C_2$-arsenobetaine). Samples were vortexed for 5 min and centrifuged at 13,000*g* for 5 min and then transferred to 96-well polypropylene microtiter plates and covered ready for analysis. Plates were stored at −20 °C and run within 12 h. Betaines have been shown to be stable in plasma and serum at 0 and 25 °C for at least 72 h [20].

To calibrate the data, aqueous standards of TMAO and glycine betaine were prepared at concentrations of 25, 50, 75, 100, and 200 μM and those of arsenobetaine, homarine, and DMSP were prepared at concentrations of 5, 10, 15, 20, and 30 μM. These standards were prepared by serial dilution of a 10-mM aqueous stock standard and stored at −80 °C in 1.5-ml Eppendorf tubes until needed. Then 50 μl of standard was added to 500 μl of extraction solvent and processed as above.

LC–MS/MS

Separation was performed on a Shimadzu Prominence HPLC system (Kyoto, Japan). The flow rate was 400 μl/min. Solvent A contained 10 mM ammonium formate, 10 mM formic acid, 50% water, and 50% acetonitrile. Solvent B contained 90% acetonitrile and 10% water. The gradient used for the analysis was as follows: 0 min of 50% A and 50% B, 6.50 min of 100% A and 0% B, and 6.60 min of 50% A and 50% B. All gradient steps were linear. Nitrogen was used as the collision gas. There was an interval of 8 min between injections. The autosampler temperature was set to 10 °C, and the injection volume was 10 μl. A Cogent Diamond Hydride silica column (100 × 2.1 mm, 4 μm, MicroSolv Technology, Eatontown, NJ, USA) was used, and the oven temperature was set at 40 °C.

Samples were measured using an MDS Sciex API 4000 tandem mass spectrometer (Applied Biosystems, Mulgrave, VIC, Australia) with a turbo ion spray (electrospray ionization) probe. Methylamines were measured in positive ion mode using multiple reaction monitoring (MRM). Mass transitions used for MRM are shown in Fig. 1. The cycle time was 450 ms, and the ion source temperature was 350 °C.

To identify compound-specific mass spectrometer parameters, a compound optimization was performed by infusing 2-μM standards of each analyte in 15 mM ammonium formate and acetonitrile (75:25 by volume) into the mass spectrometer using the syringe pump at a flow rate of 10 μl/min. Compound-specific parameters are given in Table 1. The HPLC system and mass spectrometer were controlled using Analyst software (Applied Biosystems). Aqueous external standards containing known concentrations of each analyte and deuterated internal standards were used to calibrate the data. Results were calculated using ratios of the peak area of the analytes to their deuterated internal standards.

Table 1
Compound-specific mass spectrometer parameters obtained for each analyte.

Analyte	Mass transition	DP	CE	CXP
Glycine betaine	118 → 59	56	27	4
D_9-Glycine betaine	127 → 68	61	27	4
TMAO	76 → 58	16	27	10
D_9-TMAO	85 → 66	91	29	2
Homarine	138 → 96	111	31	10
D_4-Homarine	142 → 100	81	31	6
DMSP	135 → 73	71	23	4
D_6-DMSP	141 → 73	56	23	4
Arsenobetaine	179 → 120	111	29	8
$^{13}C_2$-Arsenobetaine	181 → 120	71	29	8

Note: DP, decoupling potential; CE, collision energy; CXP, collision cell exit potential (units in electron volts, eV).

Precision and accuracy

Six batches of five replicates of bovine serum with both a low added level of the analytes and a high added level of the analytes were run by LC–MS/MS. Glycine betaine, TMAO, AsB, DMSP, and homarine were measured in the replicates, and the within- and between-batch coefficients of variation (CVs) were calculated for each compound. The recoveries of added analytes were measured to provide an indication of the accuracy of the method.

Linearity

Five different concentrations of glycine betaine, TMAO, AsB, homarine, and DMSP were added to bovine serum, and each was assayed in triplicate. The added concentrations were plotted against the observed concentrations. The linearity was determined using linear regression analysis.

Seal plasma

Plasma taken from a Weddell seal was used to test the method and evaluate matrix effects. Here, 100 μM of each analyte was added. The samples were run by LC–MS/MS, and recoveries were calculated.

Results

LC–MS/MS

The Cogent Diamond Hydride silica column separated the analytes sufficiently for detection by MS/MS (Fig. 2). Although DMSP and TMAO were coeluting, the monitoring of the different mass transitions ensured that they were not present in the same chromatograms.

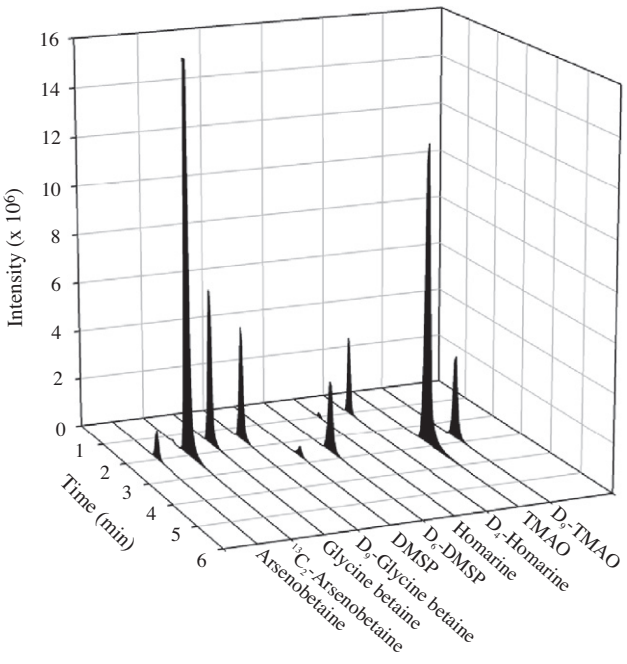

Fig.2. LC–MS/MS multiple reaction monitoring chromatograms of osmolytes (100 μM TMAO and glycine betaine, 20 μM AsB, DMSP, and homarine) and deuterated internal standards (10 μM) in bovine serum.

Table 2
Results of precision and recovery study of low and high added levels of osmolytes in bovine serum.

Analyte	Mean[a] (μmol/L)	Within-batch CV	Between-batch CV	Recovery (%)	LOD (μmol/L)
Glycine betaine					
Low	245	3.6	1.9		
High	372	4.0	2.8	85	0.04
TMAO					
Low	70	4.4	2.4		
High	200	4.6	0.9	87	0.02
Arsenobetaine					
Low	9	3.5	6.0		
High	28	3.6	2.9	93	0.03
DMSP					
Low	9	4.0	2.7		
High	28	4.6	1.8	95	0.10
Homarine					
Low	9	8.1	3.7		
High	26	7.7	4.5	86	0.12

[a] Mean (of triplicates) baseline concentrations in bovine serum without added levels of the analytes were 187 μmol/L glycine betaine and 14.2 μmol/L TMAO. AsB, DMSP, and homarine were not detected.

Precision and recovery

The results of the precision and recovery study for each osmolyte are shown in Table 2. Precision and recovery were investigated at low and high concentrations of 50 and 200 μM for TMAO and glycine betaine and of 10 and 30 μM for AsB, DMSP, and homarine. The within-batch CVs ranged from 3.5 to 8.1, and the between-batch CVs ranged from 0.9 to 6.0 (Table 2). The analytical recovery was between 85% and 95%. Initial recoveries were high for AsB, homarine, and DMSP (up to 130%) in bovine serum but were low for AsB and homarine (68%) in seal plasma. The assay was improved by using deuterated internal standards for these compounds.

Linearity

The assay was linear up to 200 μM for TMAO and glycine betaine and up to 30 μM for AsB, DMSP, and homarine (Fig. 3). Mammals have endogenous glycine betaine and TMAO in plasma, which explains the high level of glycine betaine present in the unspiked sample (Fig. 3A). The limits of detection (LODs, signal-to-noise = 3) ranged from 0.02 μM for TMAO to 0.12 μM for homarine (Table 2).

Seal plasma

Baseline levels of osmolytes in Weddell seal plasma were 20 μM glycine betaine, 80 μM TMAO, 0.8 μM arsenobetaine, and 0.5 μM homarine. DMSP was not detected. Mean recoveries of osmolytes added were 106% glycine betaine, 89% TMAO, 94% AsB, 91% homarine, and 101% DMSP.

Discussion

We have presented an LC–MS/MS method that can measure glycine betaine, TMAO, AsB, DMSP, and homarine simultaneously in serum within an 8-min run time with a throughput of approximately 200 samples over a 24-h time period. To our knowledge, this is the first study to measure homarine and AsB using LC–MS/MS and the first to use D_4-homarine and $^{13}C_2$-arsenobetaine as internal standards. Previously, Holm and coworkers [20] measured choline, betaine, and *N*,*N*-dimethylglycine (DMG) by MS/MS after separation on a normal-phase silica column. We observed sharper peaks with the Cogent Diamond Hydride column than with an unmodified silica column of similar dimensions. Reasonable separations of osmolytes were also achieved using titania and strong cation exchange stationary phases. To achieve good recoveries using LC–MS/MS methods, it is important to have isotopic internal standards for each analyte [21]. We initially obtained unacceptably high recoveries for AsB, homarine, and DMSP in bovine serum but obtained low recoveries in seal plasma. Matrix effects in biological samples affect the ionization of analytes in the mass spectrometer, and these problems were resolved by using deuterated internal standards for these compounds.

Measuring a range of different potential markers increases the information obtained in several ways; in the planned study, the components being measured not only come from different prey species but also will have different biological half-lives. Glycine betaine is metabolized in mammals by betaine-homocysteine methyltransferase (BHMT), and the increase following feeding is short-lived [26] even though it is not excreted in the urine [27]; DMSP especially is rapidly metabolized by BHMT and would be expected to have a short biological half-life [19,28]. AsB enters tissues as a glycine betaine analog and is not a substrate for BHMT [19] and has a long half-life [5]. Homarine is not known to be metabolized by mammals, and there currently are no data on its clearance by mammals.

These osmolytes (except for glycine betaine) will be present in seal plasma only if they ingest a certain type of prey. The presence of TMAO in the plasma for the single Weddell seal that we analyzed indicates that the seal was most likely consuming fish. However, the presence of homarine also suggests a contribution of cephalopods given that homarine is found in squid and octopus [12,29] and Weddell seals have been shown to feed on cephalopods in McMurdo Sound, Antarctica [30]. Because AsB is ubiquitous in marine organisms, it is likely to be present in seal blood regardless of what type of prey a seal is eating. DMSP was the only analyte that was not detected. Because DMSP is accumulated primarily by filter feeders, it is possible that the Weddell seal we sampled was not feeding on these types of organisms, but it is also possible that DMSP had already cleared from the plasma given its short half-life or was below the LOD.

This assay also has potential uses for measuring osmolytes in blood plasma, tissues, urine, and the diet for physiological, nutritional, and clinical studies. Glycine betaine is an important osmolyte in most animals and many plants, and it also has an important role in homocysteine metabolism in mammals [31,32]. There is an increasing interest in the importance of dietary betaine [18]. TMAO has recently been implicated in vascular disease [33] and is measured along with trimethylamine for diagnosing fish odor syndrome [34]. Arsenobetaine is excreted rapidly in human

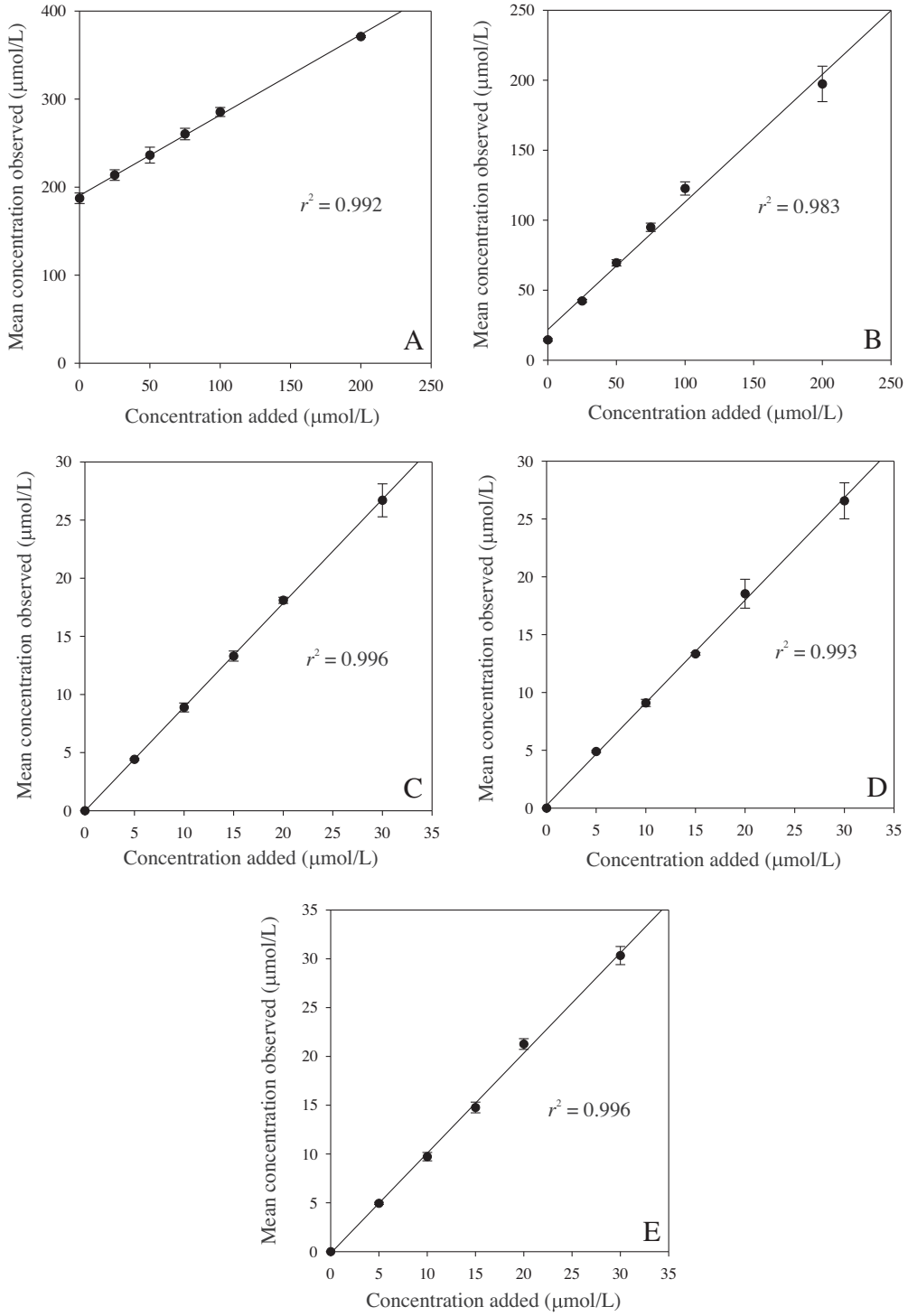

Fig.3. Glycine betaine (A), TMAO (B), AsB (C), homarine (D), and DMSP (E) added to bovine serum measured by LC–MS/MS.

urine after seafood consumption and is generally thought to be nontoxic [35], and measuring it is a definitive method to distinguish between nontoxic cationic arsenic and toxic anionic forms of arsenic. DMSP is important in environmental chemistry as the source of dimethylsulfide (DMS), the "smell of the sea," that is also formed rapidly after animal death, leading to an undesirable product through foul odor/flavor production [36]. Because most studies measure DMSP by converting it first to DMS [15,16], this method would allow direct quantification of DMSP in biological samples. This assay can also be applied to measure betaine and other analytes such as proline betaine, trigonelline, and choline in foods [37]. Thus, this assay will have a wide range of potential applications in addition to our objectives to determine the feeding behavior of Weddell seals [5].

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