Isotope Dilution LC-MS/MS Method for Glycine Betaine in Manila Clam (Tapes philippinarum)

Published Version:

Farabegoli F., Zironi E., Gazzotti T., Badiani A., Medina I., Pagliuca G. (2019). Isotope Dilution LC-MS/MS Method for Glycine Betaine in Manila Clam (Tapes philippinarum). FOOD ANALYTICAL METHODS, 12(6), 1448-1455 [10.1007/s12161-019-01469-9].

Availability:

Published:

DOI: http://doi.org/10.1007/s12161-019-01469-9

Terms of use:

Farabegoli, F., Zironi, E., Gazzotti, T. *et al.* Isotope Dilution LC-MS/MS Method for Glycine Betaine in Manila Clam (*Tapes philippinarum*). *Food Anal. Methods* **12,** 1448–1455 (2019).

Food Analytical Methods

Isotope dilution LC-MS/MS method for glycine betaine in Manila clam (Tapes philippinarum)

--Manuscript Draft--

Manuscript Number:	
Full Title:	Isotope dilution LC-MS/MS method for glycine betaine in Manila clam (Tapes philippinarum)
Article Type:	Brief Communication
Keywords:	Glycine betaine; Manila clam; UHPLC-MS/MS; validation; endogenous compound; HILIC
Corresponding Author:	Teresa Gazzotti Universita degli Studi di Bologna Scuola di Agraria e Medicina Veterinaria ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universita degli Studi di Bologna Scuola di Agraria e Medicina Veterinaria
Corresponding Author's Secondary Institution:	
First Author:	Federica Farabegoli, Ph.D.
First Author Secondary Information:	
Order of Authors:	Federica Farabegoli, Ph.D.
	Elisa Zironi
	Teresa Gazzotti
	Anna Badiani
	Isabel Medina Méndez
	Giampiero Pagliuca
Order of Authors Secondary Information:	
Funding Information:	
Abstract:	Glycine betaine (GB) is a natural compound with demonstrated metabolic functions in mammals and beneficial effects on human body: it protects cells under osmotic stress, as organic osmolyte, and acts as methylating agent in several vital biochemical pathways. The main dietary sources of GB are vegetables, such as cereals, spinach, chard and beetroot, above all; shellfish is one of the richest animal sources. A simple method for the quantification of GB in the edible portion of Manila clams (Tapes philippinarum) was set up, using ultra performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). Isotope-labelled internal standards were used during sample extraction, in order to ensure an accurate quantification. The method was successfully validated following the Commission Decision 657/2002/EC as guideline; since GB is an endogenous compound, present in very high concentrations in the target matrix, appropriate adjustments were taken. The method developed reported very satisfying performances in terms of linearity, trueness and precision; moreover its applicability was demonstrated on commercial pools of Manila clams.

Federica FARABEGOLI[a1], Elisa ZIRONI[a], Teresa GAZZOTTI[a*], Anna BADIANI[a], Isabel MEDINA[b], Giampiero PAGLIUCA[a]

Isotope dilution LC-MS/MS method for glycine betaine in Manila clam (*Tapes philippinarum*)

[a] Department of Veterinary Medical Sciences
Alma Mater Studiorum – University of Bologna
Via Tolara di Sopra, 50 – 40064 Ozzano Emilia (BO), Italy
farabegolif@gmail.com; elisa.zironi@unibo.it; anna.badiani@unibo.it; giampiero.pagliuca@unibo.it

[b] Instituto de Investigaciones Marinas del CSIC
Eduardo Cabello 6, 36208 Vigo (Pontevedra), Spain
medina@iim.csic.es

[*]Corresponding author: Teresa GAZZOTTI
Department of Veterinary Medical Sciences
Alma Mater Studiorum – University of Bologna
Via Tolara di Sopra, 50 – 40064 Ozzano Emilia (BO), Italy
Tel. +39 051 2097012
teresa.gazzotti@unibo.it

[1]ANFACO-CECOPESCA
Estrada Colexio Universitario 16, 36310 Vigo (Pontevedra), Spain

Abstract

Glycine betaine (GB) is a natural compound with demonstrated metabolic functions in mammals and beneficial effects on human body: it protects cells under osmotic stress, as organic osmolyte, and acts as methylating agent in several vital biochemical pathways.

The main dietary sources of GB are vegetables, such as cereals, spinach, chard and beetroot, above all; shellfish is one of the richest animal sources. A simple method for the quantification of GB in the edible portion of Manila clams (*Tapes philippinarum*) was set up, using ultra performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). Isotope-labelled internal standards were used during sample extraction, in order to ensure an accurate quantification.

The method was successfully validated following the Commission Decision 657/2002/EC as guideline; since GB is an endogenous compound, present in very high concentrations in the target matrix, appropriate adjustments were taken. The method developed reported very satisfying performances in terms of linearity, trueness and precision; moreover its applicability was demonstrated on commercial pools of Manila clams.

Keywords

Glycine betaine; Manila clam; UHPLC-MS/MS; validation; endogenous compound; HILIC

Chemical compounds

Glycine betaine (PubChem CID: 248); Betaine-(*trimethyl*-D_9) hydrochloride (PubChem CID: 16213877)

1. Introduction

Glycine betaine (GB) is a small natural compound, first isolated in the late nineteenth century from the juice of sugar beets (*Beta vulgaris*), hence the name "betaine" (De Zwart et al. 2003). The chemical structure of this molecule derives from the methylation of glycine, which forms a zwitterionic compound with a permanent cationic functional group (De Zwart et al. 2003; Naresh Chary et al. 2012). This feature makes GB a highly polar but neutral compound, able to retain its positive charge also at high pH (Lever and Slow 2010).

The GB intake could derive directly from the diet or from endogenous synthesis through the oxidation of choline by a two-step enzymatic process (Lever and Slow 2010). GB can be naturally found in a variety of food: the richest animal source is represented by shellfish (Zeisel et al. 2003), while the GB most abundant plants(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003)(De Zwart et al. 2003) are grass family (particularly wheat) and other belonging to the beet family, such as spinach, chard and beetroot (Lever and Slow 2010; Hefni et al. 2015)(Lever and Slow 2010; Hefni et al. 2015).

Levels of GB introduced in the diet can also vary significantly according to the type of cooking methods: due to its high water-solubility, huge losses in GB levels are expected to occur after boiling processes (De Zwart et al. 2003), while baking treatments are not expected to lower the GB content of the meal, since the internal temperature (generally below 200°C) do not induce GB degradation (European Food Safety Agency 2005).

A recommended daily intake for GB has not been established yet; an average dietary intake has been calculated to be between 100 and 300 mg/day. Higher plasma GB levels are found to be related to the intake of foods rich in nutrients as complex carbohydrates and fibre (Ueland 2011); consumers can enhance significantly GB intake by increasing shellfish consumption (De Zwart et al. 2003).

GB has two main physiologic roles in human health: it protects cells under osmotic stress, as organic osmolyte, and acts as methylating agent in several vital biochemical pathways. GB permits to maintain optimal cell turgor without affecting cellular functions, being highly compatible with enzymes and hormones activity. The enzyme "betaine homocysteine methyl transferase" (BHMT) transfers a methyl group from GB to homocysteine and regulates GB concentration controlling its partitioning for the use as an osmoprotectant or as a methyl donor (Ueland 2011). The processes of methylation of homocysteine to methionine would contribute to nucleic acid, protein and lipid synthesis and to the occurrence of several consequent beneficial effects (European Food Safety Agency 2005).

The methylation of homocysteine to methionine promotes the therapeutic role of GB in patients affected by homocysteinemia and explains its important role in human health (Craig 2004). In 2011 the European Food Safety Agency (EFSA) confirmed the scientific evidence of the beneficial effects of GB on cardiovascular system by maintaining a physiologic homocysteine metabolism, achievable with the daily intake of at least 1.5 g of GB (European Food Safety Agency 2011).

GB is also considered a lipotropic factor and contributes to the prevention or reduction of lipid accumulation in the liver, especially due to ethanol-induced fatty infiltration (European Food Safety Agency 2005).

Moreover, methyl group availability influences metabolic activities correlated to gene transcription and genomic stability (Ueland 2011). A diet rich in methylating agents (such as GB) has been reported to protect from colorectal cancer (Bae et al. 2014) and to possibly reduce risk of breast cancer (Lever and Slow 2010). Methyl groups provided by GB can also promote the synthesis of creatine, that contributes to enhance athletic performances in humans (Lever and Slow 2010; Cholewa et al. 2014). At the end, GB plays also an important role in human development, from all stages of gestation to early infancy; since choline catabolism to GB is irreversible, an adequate maternal intake of GB can ensure choline supply for phospholipid and neurotransmitter synthesis to guarantee an optimal foetal and child neurodevelopment (Lever and Slow 2010). It is increasingly well known that this compound plays an important role in human health and is essential for normal body function; consequently, deficiency in its supply and metabolism would lead to pathological consequences.

The small dimensions, the high polarity and the permanent cationic moieties of GB makes mass spectrometry (MS) an attractive choice for its analysis (Airs and Archer 2010; Li et al. 2010; Naresh Chary et al. 2012). Most of the publications available in literature reported methods for the detection and the quantification of GB in biological liquid matrices (generally plasma, serum and urine); only few methods have been developed for food or other biological matrices. To the best of our knowledge, only few studies reported data of GB in clams*:* de Zwart *et al.* (2003) used high performance liquid chromatography (HPLC) coupled with UV detector for a large-scale monitoring on GB content in foods; more recently, Ji *et al.* (2015) used *Tapes philippinarum* as bioindicator for metabolomics assays on toxicological status of clams, using nuclear magnetic resonance spectroscopy. Li *et al*. (2010) developed and validated a method using HPLC coupled with high resolution mass spectrometry for the analysis of GB in coral tissue; they successfully tested the methods on clams, but any validation procedure on this matrix was lately published. The aim of this work was to develop an ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) method for the quantification of GB in *Tapes philippinarum*, in order to contribute to the assessment of the health benefits of seafood consumption. The method proposed was validated considering the guidelines of the Commission Decision 2002/657/EC (Commission Decision 2002), adequately adapted for the analysis of an endogenous compound present in very high concentrations in matrix. Actually this is the first UPLC-MS/MS validated method for the analysis of GB in Manila clam tissue.

2. Material and methods

2.1 Reagents and chemicals

Betaine BioUltra (purity ≥ 99.0%) and its deuterated analogue internal standard (IS), betaine-(*trimethyl*-D_9) hydrochloride (D_9-BET) (purity ≥ 98.0%), were purchased by Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol, formic acid and ammonium acetate, all of LC-MS grade, were acquired by Sigma-Aldrich (St. Louis, MO, USA); dichloromethane, of laboratory grade, from Merck (Darmstadt, Germany) and Ultrapure water was freshly produced from a Milli-Q® water purification system (Merck, Darmstadt, Germany).

2.2 Sample preparation

Samples of *Tapes philippinarum* were purchased in a local market in Bologna (Italy), suppressed by congelation and stored at -18 °C. Before the extraction procedures, the tissues of 20 frozen Manila clams were minced and homogenised in a glass tube with Ultraturrax (IKA, Staufen im Breisgau, Germany), to obtain a pool of samples. 50 mg of the pooled clams, 10 mL of distilled water and 40 µL of D_9-BET (100 µg/g) were placed in a falcon tube and mixed with Vortex mixer (Velp Scientifica, Monza, Italy) for about 1 minute. The sample was then centrifuged for 5 minutes at 8000 rpm at 25°C and 5 mL of supernatant were transferred in a new falcon tube. The extract was purified vortexing with 3 mL of dichloromethane for 1 minute. The complete separation in two phases was obtained by centrifugation, at the same conditions described above; protein precipitation was then achieved adding 4 mL of acetonitrile LC-MS/MS to 1 mL of the upper aqueous phase, mixing and centrifuging in the same conditions. Finally, 1 mL of supernatant was transferred in a glass vial, ready for the injection in the UPLC-MS/MS.

2.3 UPLC-MS/MS analysis

GB analysis were performed on a Waters Acquity UPLC® binary pump equipped with a Waters Acquity UPLC® BEH HILIC (1.7 µm 2.1 x 50 mm), fitted with a Waters VanGuard™ guard column with the same phase (5 x 2.1 mm, 1.7 µm) (Waters Corporation, Milford MA, USA). The injection volume was 1 µL and the column temperature was set to 40 °C. The analyses were carried out under programmed conditions, at constant flow rate of 0.6 mL/min, employing ammonium acetate solution 10 mM acidified with 0.05% formic acid (A), and acetonitrile (B) as mobile phase. The total run time was 4 minutes, and the program started with 1 min of 5% A in isocratic conditions; then the percentage of A gradually increased to 60% in 1 min and hold for 0.5 min. Finally, the initial conditions were restored to 5% A in 0.5

min and hold for further 1 min, to equilibrate the column. Samples were maintained at 20°C in the autosampler. The chromatographic system was coupled with a Waters Quattro Premier XE™ triple quadrupole mass spectrometer equipped with an ESCi™ Multi-Mode Ionization Source (Waters Corporation, Milford MA, USA) operating in positive electrospray ionization (ESI+) mode. Analysis were performed in MRM (multiple reaction monitoring) mode, monitoring two transitions for GB and two for the deuterated IS; the selected transitions (and the relative values of CV and CE, respectively) were the following: 118.2 > 59.3 *m/z* (38 V, 16 eV) and 118.2 > 58.3 *m/z* (38 V, 23 eV) for GB; 127.2 > 68.4 *m/z* (38 V, 18 eV) and 127.2 > 68.4 *m/z* (38 V, 27 eV) for D_9-BET. Argon was used as collision gas at a flow of 0.35 mL/min. The instrument settings were: capillary voltage 4.00 kV, source temperature 140 °C, desolvation temperature 325 °C, cone gas 150 L/h and desolvation gas 550 L/h. A Waters MassLynx™ 4.1 software (Waters Corporation, Milford MA, USA) was used to acquire and process data.

2.4 Method validation

The described method was validated considering the guidelines of the Commission Decision 2002/657/EU (Commission Decision 2002). The use of a deuterated IS ensured the quality of data and GB was quantified considering the ratio between its area and that of the IS on matrix-matched calibration curves.

Two working solutions were prepared, one for GB and one for the IS, at a concentration of 5000 µg/mL and 100 µg/mL in acetonitrile:water = 95:5, respectively. Linearity was evaluated by matrix-matched calibration curves, prepared each day of validation, spiking 50 mg of clam tissue with a fixed amount of IS (40 µL of IS working solution) and 4 increasing levels of GB, as reported in Table 1. The limit of quantification (LOQ) of the method was identified as the lowest point of the calibration curve (2.5 µg/mg). To establish the average endogenous content of GB in Manila clams, an appropriate number of representative blank samples (a total of twenty) were processed and analysed (Commission Decision 2002). The percent standard deviation of the data obtained by the analysis of 20 samples (fortified with the fixed amount of the IS) resulted below 15%; therefore, the mean value of the responses was used to be matched with the zero value of the calibration curve.

Trueness and precision of the method were calculated by the analysis of matrix-matched quality control (QC) samples, realised in six replicates for three day of validation: clams samples were fortified with the fixed amount of IS, and at 4 µg/mg (QC low), at 6 µg/mg (QC medium) and at 8 µg/mg (QC high) of GB.

Trueness, expressed as bias, is the relative difference between the mean measured value and the spiked concentration, and for these concentrations the bias values should be in the range between -20% and +10% (Commission Decision 2002).

Precision was measured as relative standard deviation to the mean (CV%). Since the mass fraction of this analyte in the target matrix, expressed as a power of 10, is -6, values of CV% in within-laboratory reproducibility conditions (different operators, different days) should be lower than 16% (Commission Decision 2002), calculated by the Horwitz equation. As far as repeatability conditions (intra-day analysis) are concerned, CV% values should be lower than 11%, which is two-thirds of the value calculated by the Horwitz equation.

The analyte stability in matrix was assayed under different storage conditions. For this purpose two samples fortified at QC medium level were prepared; one was maintained for 24 h in autosampler (bench-top stability) at 20°C and one was subject to freeze-thaw cycles of 1, 2 ,4 and 20 weeks. The acceptance criteria for all stability tests were an accuracy of within ±15% (European Medicines Agency 2011). Before the analysis of the frozen samples, the vials containing the extracts were left at room temperature for two hours to obtain a complete thaw of the sample.

The presence of a carry-over of the target molecule in the system was assessed by means of six consecutive injections of a solution free of the target compounds, consisting of water:acetonitrile 50:50, after the injection of a calibration curve. The intensity of eventual signals should not be greater than the 20% of those obtained by the injection of the lower point of the calibration curve; likewise, concerning the IS, the signal should not overweight the 5% (European Medicines Agency 2011).

3. Results and discussion

3.1 Chromatographic method development

The chromatographic technique, characterised by the use of a normal stationary phase in combination with a reverse mobile phase, is the “Hydrophilic Interaction Liquid Chromatography” (HILIC) (Zhao et al. 2011). This technology provides good retention of strongly polar molecules employing high percentages (more than 50%) of organic solvents (often acetonitrile) in the mobile phase, which are especially compatible with ESI source, due to their low viscosity. HILIC technique therefore provides high performances in terms of sensitivity, selectivity and separation efficiency (Jandera 2011; Zhao et al. 2011). Several authors in the last years exploited this technology for the quantification of GB in biological liquid matrices, including plasma (Bruce et al. 2010; Kirsch et al. 2010), serum (Steuer et al. 2016), urine (Ocque et al. 2015; Zhao et al. 2015), amniotic fluid and cerebrospinal fluid (Kirsch et al. 2010), but also in foods and tissues (Zhao et al. 2011; Xiong et al. 2012), seaweeds(MacKinnon et al. 2010), and cereal products (Bruce et al. 2010; Ross et al. 2014).

Also in the present work, the interactions between the HILIC stationary phase and GB and IS allowed a successfully retention of analytes. The mobile phases generally used for GB and related compounds analysis are acetonitrile and aqueous solution with 5-20 mM of ammonium formiate or acetate, acidified with formic or acetic acid, to reach a pH between 3 and 3.5. In order to optimize the chromatographic conditions for GB, 10 mM of ammonium acetate and 10 mM of ammonium formiate in aqueous solution were tested, but no difference in terms of sensitivity between the two buffers were noted. Regarding pH optimisation, better performances have been reached at values of 3.6, obtained adding 0.05% of formic acid, according to the concentrations used in other studies (Bruce et al. 2010; Kirsch et al. 2010; Ocque et al. 2015).

The use of optimised mobile phases and programmed conditions employing increasing concentration of water to elute the analytes allowed to achieve narrow and symmetric peak shapes for both GB and IS, in a very short chromatographic run (4 min, including column re-equilibration). The high percentages of acetonitrile (never below 40%) in the ramp also provided high performances in terms of sensitivity due to its high compatibility with ESI source.

3.2 Extraction procedure optimisation

This extraction procedure is the result of the optimization of two published methods, developed by de Zwart *et al.* (2003) and Hefni *et al.* (2015), for the determination of GB in food. The procedure consisted in a simple and rapid liquid-liquid extraction (LLE) method using water as solvent, exploiting the highly water-solubility of the analyte (Lever and Slow 2010). A purification step with dichloromethane resulted necessary to remove non-polar analytes, which could interfere during GB ionization (e.g. phospholipids). Then, a SPE (solid-phase extraction) purification step could be avoided, making the method described simple, rapid and cheap.

This extraction procedure was compared with those employed by Koc, *et al*. (2002), Zhao *et al.* (2011) and Xiong *et al.* (2012), to extract GB from foodstuff applying modified versions of the Bligh and Dyer method (Bligh and Dyer 1959). Methanol, as extraction solvent, has been largely used for the analysis of GB in foods (Zeisel et al. 2003; Bruce et al. 2010; Ji et al. 2015; Stiboller et al. 2015) or other biological matrices (Li et al. 2010; MacKinnon et al. 2010; Pekkinen et al. 2013; Wang et al. 2014); however, for the purposes of this work, the use of methanol resulted not suitable, since it prevents the optimal two-phases separation between water and the non-polar solvent used for the purification step.

Several tests were carried out to establish the amount of sample to be processed; 50 mg of clam pulp were selected as the optimum compromise to obtain an easy sample handling and reasonable concentration of endogenous analyte. The extraction efficiency of different solvent volumes (3, 5 and 10 mL) had also been tested: the addition of 10 mL (suggested by Hefni *et al.* 2015) of water resulted the best choice, as the great analyte content in the target matrix allowed high dilution rates. Moreover, the extension of the extraction time to 30 minutes or the addition of ultrasonication steps did not lead to any notable improvements in recovery rates of the extraction procedure. The sensibility of the technology adopted allowed a further dilution (4:1) with acetonitrile, at the end of the extraction process, with the aim to promote the precipitation of proteins and the dilution of salts; the increasing in the percentage of organic phase in the sample also favours the ionisation in ESI source.

3.3 Validation of the method

Validation of the described method for the quantification of GB in *Tapes philippinarum* was performed considering the guidelines of the Commission Decision 2002/657/EC (Commission Decision 2002), with the application of appropriate adjustments, due to the unavoidable presence and the high levels of GB in *Tapes philippinarum*. As a consequence, it was impossible to find a blank matrix; anyway the method developed did not involve the use of any surrogate matrix for the validation procedures.

The linearity was evaluated by the injection of matrix-matched calibration curves, prepared as described in Table 1; the regression coefficients (R^2) resulted always above 0.99 for each curve, demonstrating a good linearity of the method.

The analysis of quality control samples, fortified as described in section 2.4, reported satisfying results. All the values of precision both in within-laboratory reproducibility conditions and in repeatability conditions (intra-day analysis), were lower than CV% values recommended by Commission Decision 2002/657/EC, and trueness was included in the range of -20% and +10%, as reported in Table 2.

The stability of the analyte in the matrix was assessed in two different storage conditions: the former for 24 h in autosampler (bench-top stability) at 20°C and the latter subjected to freeze-thaw cycles of 1, 2, 4 and 20 weeks.

The bench-top assay at 20 °C showed a loss of GB concentration of about 25% after 24 hours and the freeze-thaw stability at -18 °C was confirmed only for two cycles (two weeks), according to the limits mentioned in section 2.4 (±15%). In general GB shows a good stability even if evaluated in different matrices such as plasma and urine (Holm et al. 2003; Kirsch et al. 2010; Xiong et al. 2012; Midttun et al. 2013), but currently no data on GB stability in extracted Manila clams have been published. Finally, the carry over for this compound was considered irrelevant because the signal at the specific retention time of GB had an acceptable intensity (below 20% of LOQ). Concerning the IS, no signal were detected at its retention time, demonstrating the absence of carry over for this compound.

The proposed method was tested on five pools from three different batch of commercial *Tapes philippinarum* samples (n = 15). The results of this test are reported in Table 3; the average level of GB measured in these samples (4.1 µg/mg) is comparable with the level (2.5 µg/mg) assessed by De Zwart *et al*. (2003).

4. Conclusions

A rapid and economic method based on UPLC-MS/MS for the detection of GB in *Tapes philippinarum* was developed. The procedure was successfully validated considering the European regulation guidelines. The method proposed results simple and rapid and shows very satisfying performances in terms of linearity, trueness and precision. Currently no other method has been developed and validated for the detection and quantification of GB in *Tapes philippinarum* by UPLC-MS/MS. The procedure proposed could be considered a practical and useful tool for the assessment of betaine in molluscs.

Acknowledgments

The authors thank Lucía Méndez and María Jesús González for their excellent techncial assistance.

References

Airs RL, Archer SD (2010) Analysis of glycine betaine and choline in seawater particulates by liquid chromatography/electrospray ionization/mass spectrometry. Limnol Oceanogr Methods 8:499–506. doi: 10.4319/lom.2010.8.499

Bae S, Ulrich CM, Neuhouser ML, et al (2014) Plasma choline metabolites and colorectal cancer risk in the Women’s Health Initiative Observational Study. Cancer Res 74:7442–7452. doi: 10.1158/0008-5472.CAN-14-1835

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–916

Bruce SJ, Guy P a., Rezzi S, Ross AB (2010) Quantitative measurement of betaine and free choline in plasma, cereals and cereal products by isotope dilution LC-MS/MS. J Agric Food Chem 58:2055–2061. doi: 10.1021/jf903930k

Cholewa JM, Guimarães-Ferreira L, Zanchi NE (2014) Effects of betaine on performance and body composition: A review of recent findings and potential mechanisms. Amino Acids 46:1785–1793. doi: 10.1007/s00726-014-1748-5

Commission Decision (2002) 2002/657/EC Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off J Eur Communities

Craig SAS (2004) Betaine in human nutrition. Am J Clin Nutr 80:539–549

De Zwart FJ, Slow S, Payne RJ, et al (2003) Glycine betaine and glycine betaine analogues in common foods. Food

Chem 83:197–204.
doi: 10.1016/S0308-8146(03)00063-3

European Food Safety Agency (2005) Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to an application concerning the use of betaine as a novel food in the EU

European Food Safety Agency (2011) Scientific Opinion on the substantiation of health claims related to betaine and contribution to normal homocysteine metabolism (ID 4325) pursuant to Article 13 (1) of Regulation (EC) No 1924/2006

European Medicines Agency (2011) Guideline on bioanalytical method validation. 1–23.
doi: EMEA/CHMP/EWP/192217/2009

Hefni M, McEntyre C, Lever M, Slow S (2015) Validation of HPLC-UV Methods for the Quantification of Betaine in Foods by Comparison with LC-MS. Food Anal Methods 9:292–299.
doi: 10.1007/s12161-015-0195-6

Holm PI, Ueland PM, Kvalheim G, Lien E a. (2003) Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. Clin Chem 49:286–294.
doi: 10.1373/49.2.286

Jandera P (2011) Stationary and mobile phases in hydrophilic interaction chromatography: A review. Anal Chim Acta 692:1–25.
doi: 10.1016/j.aca.2011.02.047

Ji C, Cao L, Li F (2015) Toxicological evaluation of two pedigrees of clam Ruditapes philippinarum as bioindicators of heavy metal contaminants using metabolomics. Environ Toxicol Pharmacol 39:545–554.
doi: http://dx.doi.org/10.1016/j.etap.2015.01.004

Kirsch SH, Herrmann W, Rabagny Y, Obeid R (2010) Quantification of acetylcholine, choline, betaine, and dimethylglycine in human plasma and urine using stable-isotope dilution ultra performance liquid chromatography-tandem mass spectrometry. J Chromatogr B 878:3338–3344.
doi: 10.1016/j.jchromb.2010.10.016

Lever M, Slow S (2010) The clinical significance of betaine, an osmolyte with a key role in methyl group metabolism. Clin Biochem 43:732–744.
doi: 10.1016/j.clinbiochem.2010.03.009

Li C, Hill RW, Jones a. D (2010) Determination of betaine metabolites and dimethylsulfoniopropionate in coral tissues using liquid chromatography-time-of-flight mass spectrometry and stable isotope-labeled internal standards. J

Chromatogr B 878:1809–1816.
doi: 10.1016/j.jchromb.2010.05.014

MacKinnon SL, Hiltz D, Ugarte R, Craft CA (2010) Improved methods of analysis for betaines in Ascophyllum nodosum and its commercial seaweed extracts. J Appl Phycol 22:489–494.
doi: 10.1007/s10811-009-9483-0

Midttun Ø, Kvalheim G, Ueland PM (2013) High-throughput, low-volume, multianalyte quantification of plasma metabolites related to one-carbon metabolism using HPLC-MS/MS. Anal Bioanal Chem 405:2009–2017.
doi: 10.1007/s00216-012-6602-6

Naresh Chary V, Dinesh Kumar C, Vairamani M, Prabhakar S (2012) Characterization of amino acid-derived betaines by electrospray ionization tandem mass spectrometry. J Mass Spectrom 47:79–88.
doi: 10.1002/jms.2029

Ocque AJ, Stubbs JR, Nolin TD (2015) Development and validation of a simple UHPLC-MS/MS method for the simultaneous determination of trimethylamine N-oxide, choline, and betaine in human plasma and urine. J Pharm Biomed Anal 109:128–135.
doi: 10.1016/j.jpba.2015.02.040

Pekkinen J, Olli K, Huotari A, et al (2013) Betaine supplementation causes increase in carnitine metabolites in the muscle and liver of mice fed a high-fat diet as studied by nontargeted LC-MS metabolomics approach. Mol Nutr Food Res 57:1959–1968.
doi: 10.1002/mnfr.201300142

Ross AB, Zangger A, Guiraud SP (2014) Cereal foods are the major source of betaine in the Western diet - Analysis of betaine and free choline in cereal foods and updated assessments of betaine intake. Food Chem 145:859–865.
doi: 10.1016/j.foodchem.2013.08.122

Steuer C, Schütz P, Bernasconi L, Huber AR (2016) Simultaneous determination of phosphatidylcholine-derived quaternary ammonium compounds by a LC–MS/MS method in human blood plasma, serum and urine samples. J Chromatogr B 1008:206–211.
doi: http://dx.doi.org/10.1016/j.jchromb.2015.12.002

Stiboller M, Raber G, Francesconi KA (2015) Simultaneous determination of glycine betaine and arsenobetaine in biological samples by HPLC/ICPMS/ESMS and the application to some marine and freshwater fish samples. Microchem J 122:172–175.
doi: http://dx.doi.org/10.1016/j.jchromb.2015.12.002

Ueland PM (2011) Choline and betaine in health and disease. J Inherit Metab Dis 34:3–15.

doi: 10.1007/s10545-010-9088-4

Wang X, Zhao P, Liu X, et al (2014) Quantitative profiling method for phytohormones and betaines in algae by liquid chromatography electrospray ionization tandem mass spectrometry. Biomed Chromatogr 28:275–280. doi: 10.1002/bmc.3018

Xiong Y, Zhao YY, Goruk S, et al (2012) Validation of an LC-MS/MS method for the quantification of choline-related compounds and phospholipids in foods and tissues. J Chromatogr B 911:170–179. doi: 10.1016/j.jchromb.2012.10.038

Zeisel SH, Mar M-H, Howe JC, Holden JM (2003) Concentrations of Choline-Containing Compounds and Betaine in Common Foods. J Nutr 133:2918–2920

Zhao X, Zeisel SH, Zhang S (2015) Rapid LC-MRM-MS assay for simultaneous quantification of choline, betaine, trimethylamine, trimethylamine N-oxide, and creatinine in human plasma and urine. Electrophoresis 36:2207–2214. doi: 10.1002/elps.201500055

Zhao YY, Xiong Y, Curtis JM (2011) Measurement of phospholipids by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry: The determination of choline containing compounds in foods. J Chromatogr A 1218:5470–5479. doi: 10.1016/j.chroma.2011.06.025

Table 1: *Calibration curve preparation for the analysis of GB*

Concentration (µg/mg)	**µL D_9-BET (100 µg/mL)**	**µL BET (5000 µg/mL)**
endogenous	40	-
2.5	40	25
5	40	50
7.5	40	75
10	40	100

Table 2: *Trueness and precision results*

Fortification level (µg/mg)	**Parameter**	**Day 1**	**Day 2**	**Day 2**	**Reproducibility (Inter-day)**
4	Precision (CV%)	3.5	6.0	5.5	4.6
	Trueness (bias%)	-2.6	0.3	-0.6	-1.0
6	Precision (CV%)	5.5	5.6	5.6	5.2
	Trueness (bias%)	3.1	-1.0	-0.4	0.6
8	Precision (CV%)	4.0	0.7	0.5	3.0
	Trueness (bias%)	3.7	-0.5	-1.0	0.7

Table 3: *GB concentration in commercial Tapes philippinarum*

Concentration (µg/mg)		
Batch 1	Batch 2	Batch 3
3.7	6.1	3.8
4.5	4.2	3.9
4.1	5.4	3.3
3.6	4.3	3.6
3.2	4.7	3.6