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

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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:

This version is available at: <https://hdl.handle.net/11585/692586> since: 2019-07-22

Published:

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

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(Article begins on next page)

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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).

The final published version is available online at: <https://doi.org/10.1007/s12161-019-01469-9>

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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 (<i>Tapes philippinarum</i>)
Article Type:	Brief Communication
Keywords:	Glycine betaine; Manila clam; UHPLC-MS/MS; validation; endogenous compound; HILIC
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Order of Authors Secondary Information:	
Funding Information:	
Abstract:	<p>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.</p> <p>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 (<i>Tapes philippinarum</i>) 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.</p> <p>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.</p>

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

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27 Abstract

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

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

36 The method was successfully validated following the Commission Decision 657/2002/EC as guideline; since GB is an
37 endogenous compound, present in very high concentrations in the target matrix, appropriate adjustments were taken.

38 The method developed reported very satisfying performances in terms of linearity, trueness and precision; moreover its
39 applicability was demonstrated on commercial pools of Manila clams.

40 Keywords

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

42 Chemical compounds

43 Glycine betaine (PubChem CID: 248); Betaine-(*trimethyl-D₉*) hydrochloride (PubChem CID: 16213877)

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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

109 endogenous compound present in very high concentrations in matrix. Actually this is the first UPLC-MS/MS validated
110 method for the analysis of GB in Manila clam tissue.

111

112 **2. Material and methods**

113 **2.1 Reagents and chemicals**

114 Betaine BioUltra (purity $\geq 99.0\%$) and its deuterated analogue internal standard (IS), betaine-(*trimethyl*-D₉)
115 hydrochloride (D₉-BET) (purity $\geq 98.0\%$), were purchased by Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile,
116 methanol, formic acid and ammonium acetate, all of LC-MS grade, were acquired by Sigma-Aldrich (St. Louis, MO,
117 USA); dichloromethane, of laboratory grade, from Merck (Darmstadt, Germany) and Ultrapure water was freshly
118 produced from a Milli-Q® water purification system (Merck, Darmstadt, Germany).

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120 **2.2 Sample preparation**

121 Samples of *Tapes philippinarum* were purchased in a local market in Bologna (Italy), suppressed by congelation and
122 stored at -18 °C. Before the extraction procedures, the tissues of 20 frozen Manila clams were minced and homogenised
123 in a glass tube with Ultraturrax (IKA, Staufen im Breisgau, Germany), to obtain a pool of samples. 50 mg of the pooled
124 clams, 10 mL of distilled water and 40 µL of D₉-BET (100 µg/g) were placed in a falcon tube and mixed with Vortex
125 mixer (Velp Scientifica, Monza, Italy) for about 1 minute. The sample was then centrifuged for 5 minutes at 8000 rpm
126 at 25°C and 5 mL of supernatant were transferred in a new falcon tube. The extract was purified vortexing with 3 mL of
127 dichloromethane for 1 minute. The complete separation in two phases was obtained by centrifugation, at the same
128 conditions described above; protein precipitation was then achieved adding 4 mL of acetonitrile LC-MS/MS to 1 mL of
129 the upper aqueous phase, mixing and centrifuging in the same conditions. Finally, 1 mL of supernatant was transferred
130 in a glass vial, ready for the injection in the UPLC-MS/MS.

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132 **2.3 UPLC-MS/MS analysis**

133 GB analysis were performed on a Waters Acquity UPLC® binary pump equipped with a Waters Acquity UPLC® BEH
134 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
135 µm) (Waters Corporation, Milford MA, USA). The injection volume was 1 µL and the column temperature was set to
136 40 °C. The analyses were carried out under programmed conditions, at constant flow rate of 0.6 mL/min, employing
137 ammonium acetate solution 10 mM acidified with 0.05% formic acid (A), and acetonitrile (B) as mobile phase. The
138 total run time was 4 minutes, and the program started with 1 min of 5% A in isocratic conditions; then the percentage of
139 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

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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 D₉-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 10^{-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 $\pm 15\%$ (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 successful 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 formate 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 formate 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 ($\pm 15\%$). 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 ($4.1 \mu\text{g/mg}$) is comparable with the level ($2.5 \mu\text{g/mg}$) assessed by De Zwart *et al.* (2003).

4. Conclusions

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

266

267 **Acknowledgments**

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

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371 **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

372
373 **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

374
375 **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